BMJ Open

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Journal:	*BMJ Open*
Manuscript ID	bmjopen-2018-024733
Article Type:	Protocol
Date Submitted by the Author:	21-Jun-2018
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Keywords:	glucose homeostasis, insulin resistance, acupuncture, metformin, life style, polycystic ovary syndrome

Acupuncture or metformin for insulin resistance in women with polycystic ovary syndrome: Study protocol of a combined multinational case-control and a randomized controlled trial

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Short title: Acupuncture or metformin for insulin resistance in PCOS

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Author Contributions

ESV, HZ, RL, ALH and JQ conceived and designed the study. ESV and JQ sought funding and ethical approval. CF, DL, WW, HW, CC, SL, ZH, HZ, XJ and ALH recruited and screened subjects, coordinated and carried out acupuncture treatment. ESV drafted the manuscript. All authors read and approved the final manuscript.

Abstract

Introduction: Polycystic ovary syndrome (PCOS) is linked to hyperinsulinemia and insulin resistance with dysfunctional glucose metabolism. Pilot studies suggests that acupuncture treatment with combined manual and low-frequency electrical stimulation (electroacupuncture, EA) of the needles decrease circulating glycated hemoglobulin (HbA1c) and homeostatic model assessment-insulin resistance (HOMA-IR). Therefore, we here aim to investigate if acupuncture treatment or metformin alone or together with life style management improve insulin sensitivity and related symptoms in overweight/obese women with PCOS.

Methods and analysis: This is a two centre multinational (Sweden and China), prospective case-control study combined with an open-labelled randomized controlled trial (RCT) with a comparative effectiveness design. Participants are randomized to one of three groups: 1) life style management alone; 2) EA 2-3 times/week during 4 months + lifestyle management; or 3) metformin, 500 mg, three/day during 4 months + lifestyle management. The primary outcome measures in the RCT are Hba1C and HOMA-IR. A total of 114 (Hba1C) or 303 (HOMA-IR) obese overweight women with PCOS will be enrolled and randomized into one of the three groups with a target power of at least 80% and 5% significance level based on two-sided tests.

Ethics and dissemination: The study is performed according to good clinical practice and conducted in accordance with the Declaration of Helsinki and has been approved by the Regional Ethical Review Board of Stockholm and of Peking University Third Hospital, China. Primary outcome data of the RCT will be published in a relevant journal together with supporting secondary outcome measurements. Further, outcome measurements will be published in separate papers as well as case-control data.

Trial registration: Clinicaltrials.gov: NCT02647827 and EudraCT: 2015-004250-18.

Key words: glucose homeostasis; insulin resistance; metformin; electroacupuncture; metformin; lifestyle

Strength and Limitations

1. A strength of this trial is that all patients will benefit from receiving treatment, all of which alone and/or in combination may offer an increased chance for improved metabolic function and reproductive health.

2. It has the potential to gain deeper insight into the pathophysiology of polycystic ovary syndrome (PCOS), and to uncover new knowledge for treatment of insulin resistant in related diseases, including obesity, insulin resistance, and type 2 diabetes (T2D).

3. The results from the present study have the potential to immediately be implemented into the healthcare system since it has previously been shown to be cost-effective and to have few negative side effects.

4. A potential limitation is that metformin might cause side-effects such as diarrhoea, nausea/vomiting, flatulence, asthenia, indigestion, abdominal discomfort and headache and acupuncture local skin irritation, discomfort, and vasovagal reactions during the procedure.

Background

Polycystic Ovary Syndrome (PCOS) is the most common endocrine and metabolic disorder in women of reproductive age and is characterized by anovulation, hyperandrogenism and metabolic dysfunction.[1] Women with PCOS have a 3 to 7-fold increased risk of developing type 2 diabetes (T2D), and with younger onset, PCOS increases cardiovascular risk factors[1-6]. Independent of body weight, insulin sensitivity is ~40% lower in women with PCOS than in healthy women, and impaired glucose regulation, insulin resistance and reduced insulin responsiveness have been attributed to defects in insulin signalling in adipocytes and skeletal muscle.[7 8] Of note, obesity aggravates all symptoms related to PCOS.

Despite detrimental impact on women's health, the aetiology of PCOS is not well understood. Genetic, epigenetic, and environmental factors have all been implicated in its development. Emerging evidence suggests that PCOS originates, at least in part, in fetal life,[9 10] and elevated maternal androgens have been implicated to play a role, however the mechanisms are largely unknown.[11 12] Of interest is that we have found that women with PCOS have multiple transcriptional and epigenetic changes in adipose tissue that are relevant for development of the disease.[13] Further, twin studies suggest that genetic influences explain >70% of PCOS pathogenesis.[14] However, whether genetic or epigenetic alterations in target tissues e.g. adipose tissue, skeletal muscle and endometrium contribute to development of metabolic disease requires further investigation.

Women with PCOS require long-term individualized treatment programs. Pharmacological treatments, including the glucose reducing drug metformin, have limitations related to adverse effects and patient compliance. Therefore, there is a need for inexpensive and easily administered treatments with few negative side-effects. Lifestyle management and metformin are the first line treatment for improving whole body glucose homeostasis and preventing type 2 diabetes (T2D).[1] Interestingly, five weeks of acupuncture with combined manual and low-frequency electrical stimulation has in a pilot study been shown to improve whole body glucose homeostasis in insulin resistant women with PCOS.[15]

Whilst pharmacological treatment strategies have shown efficacy, importantly, there is a need for Comparative Effectiveness Research (CER) to strengthen the evidence base for clinical and policy decision-making [16]. Therefore we aim to compare the effect of pharmacological first-line treatment, metformin, with a non-pharmacological treatment strategy, acupuncture, with lifestyle management for improvement and prevention of metabolic dysfunction and related symptoms in insulin resistant women with PCOS.

We hypothesize that acupuncture is equally effective as metformin (both treatments combined with lifestyle management) in improving whole body glucose homeostasis in insulin resistant women with PCOS, and that both are superior to lifestyle management alone. Although equally effective (acupuncture and metformin), we hypothesize that acupuncture is associated with less negative side-effects. We also hypothesize that these treatments have the potential to restore epigenetic and molecular alterations in target tissues (endometrial-, adipose-, and skeletal muscle tissue) and thus have the potential to improve and potentially prevent the development of metabolic alterations including T2D.

Therefore, the purpose of this study is twofold, first we aim to gain deeper insight into the pathophysiology of PCOS in a case-control study by comparing women with PCOS with women without PCOS matched for age, weight and BMI in primary and secondary outcome variables, and secondly we aim to perform a prospective RCT of women with PCOS, comparing the effectiveness of lifestyle management alone, and in combination with acupuncture or metformin treatment on whole body glucose homeostasis, with the ultimate goal to prevent the development of type 2 diabetes.

Study Objectives

Primary Objective

1. To determine the clinical effectiveness of 4 months of 1) electroacupuncture + lifestyle management and 2) metformin + lifestyle management, compared to 3) lifestyle management only for improvement of insulin sensitivity as measured by homeostatic model assessment (HOMA)-IR, and by glucose regulation (assessed by analyzing Hba1c levels).

Secondary Objectives

1. To evaluate changes in secondary metabolic measures including the insulin response to glucose assessed by calculating the area under the curve ($AUC_{insulin}$) during the oral glucose tolerance test (OGTT), fasting insulin, glucose, and , calculation of HOMA-B (*i.e.* the Islet β-cell function) and the assessment of the and lipid profile, body size and proportions and body fat distribution.
2. To determine changes in gene expression and DNA methylation profiles related to insulin sensitivity in fat, muscle and endometrial tissue biopsies, and biomarkers in whole blood.

3. To evaluate endocrine measures including menstrual pattern and ovulation frequency, circulating hormones (e.g. sex steroids, AMH, gonadotropins), and excretion of metabolites of sex steroids in blood
4. To determine changes in women's health related quality of life (HRQoL), symptoms of anxiety and depression, dieting and eating patterns, and negative side-effects.

Methods and Analyses

Study design

This is a two centre multinational prospective trial with a prospective case-control part and an open-labelled RCT with a comparative effectiveness design. The interventions to be tested are 1) Electroacupuncture during 4 months + lifestyle management; 2) Metformin during 4 months + lifestyle management; or 3) Lifestyle management alone which will be available for participants in all three groups. Participants will be enrolled at Karolinska Institutet and Karolinska University Hospital, Stockholm, Sweden and at Peking University Hospital, Beijing China respectively.

Patient and Public Involvement

Patients and or public was not involved in the design of this study.

Participants

Eligible women will be identified by their clinician, or by local newspaper advertisements, and invited to participate in the trial. Each participant will be given written and oral information and asked for her signed informed consent to be randomized and followed-up by research staff. The case-control part of the study equals baseline measurements for women with PCOS and controls. If a patient do not adhere to the frequent treatment this will be clearly tracked as the treatment may still be effective.

Inclusion criteria – women with PCOS

1. Age 18 to 40 years
2. Body mass index (BMI) ≥25 to ≤40 given that 95% of all women with PCOS with a BMI ≥25 are insulin resistant.[17 18]
3. PCOS diagnosis according to Rotterdam criteria 2003 [19], with at least two of the following three symptoms: Clinical and/or biochemical signs of hyperandrogenism (hirsutism or acne); oligo/amenorrhea; and/or polycystic ovaries (PCOS). Biochemical hyperandrogenism is defined by total testosterone >1.2 nmol/L or a free

androgen index (FAI)>5.[20] Hirsutism is defined as a self-reported Ferriman-Gallwey (FG) score ≥8 (≥5 Asian).[21 22] Acne is defined by a positive response to the question *Do you have acne?* Oligomenorrhea is defined as an intermenstrual interval >35 days and <8 menstrual bleedings in the past year. Amenorrhea as <3 cycles per year. PCO is defined by transvaginal ultrasound with ≥12 follicles 2–9 mm and/or ovarian volume ≥10 ml in one or both ovaries.

4. Willing to sign the consent form.

Inclusion criteria – controls

Controls should have BMI ≥25 to ≤40, regular cycles with 28 days ± 2 days, and no signs of hyperandrogenism. They are excluded if they have menstrual irregularities, signs of hyperandrogenism (FG >4), or evidence of PCO morphology on ultrasound.

Exclusion criteria for all women

1. Age >40
2. Exclusion of other endocrine disorders such as non-classic congenital adrenal hyperplasia (17-hydroxyprogesterone < 3nmol/L), androgen secreting tumors or suspected Cushing's syndrome.
3. Having known renal disease (creatinine clearance < 60 mL/min), hepatic insufficiency, autoimmune disorders or cancer.
4. Any acute condition with potential to alter renal function or cause tissue hypoxia.
5. Type I diabetes.
6. Pharmacological treatment (cortizon, antidepressant, other antidiabetic treatment such as insulin and acarbose, hormonal contraceptives, hormonal ovulation induction or other drugs judged by discretion of investigator) within 12 weeks. Depo Provera or similar within 6 months.
7. Hypersensitivity to metformin hydrochloride or to any of the excipients.
8. Blood pressure >160 / 100 mmHg
9. Pregnancy or breastfeeding the last 6 months
10. Acupuncture the last 2 months
11. Daily smoking and alcoholic intake
12. Language barrier or disabled person with reduced ability to understand the information given.

In total 50 controls will be matched at baseline (age, weight and BMI) to women with PCOS. Controls will undergo screening and baseline visit, but will not be randomized to any treatment.

Interventions

Participants fulfilling the inclusion criteria will be randomized to one of three groups after baseline measurements:

1. Electroacupuncture 2-3 times/week during 4 months + lifestyle management
2. Metformin, 500 mg, three times/day during 4 months + lifestyle management
3. Lifestyle management alone which will be available for participants in all three groups.

Lifestyle management: All women will receive lifestyle management instructions at the baseline visit, before randomization. The lifestyle management involves one initial counselling session in connection with the baseline visit, which includes information about the importance of weight management, healthy diet and physical activity. Focus will be on the importance of physical activity. Each participant will receive a book with lifestyle advice about weight reduction, maintenance and physical activity. All participants will receive a text message once weekly to respond number of step during the last week and if they have had any menstrual bleedings. Once every fourth week, study coordinator will call to the participant and ask about number of step last week, menstruation and compliance and side-effects.

Electroacupuncture: Women randomized to receive acupuncture will start their treatment within one week after baseline measurements. The rationale of the acupuncture protocol is based on Western Medical Acupuncture theories. We will use a fixed acupuncture protocol following the two pilot studies: ClinicalTrial.gov NCT01457209 and NCT02026323 with two modifications. First, the treatment period will be 16 weeks (i.e. 4 months) compared to 5 weeks and 6 months in the previous pilot studies. Second, the treatment frequency will be 2 to 3 times per week during 16 weeks, i.e. in total 32 to 48 acupuncture treatments over 16 weeks. The rational for these changes is that the procedure is time-consuming for the patients and this will increase the feasibility and likely reduce the number of dropouts. Acupuncture treatment will be given by registered physiotherapists or medical doctors educated in theoretical and practical acupuncture and trained to follow the fixed protocol.

Disposable, single-use, sterilized CE marked needles made of stainless steel, 0.25 x 30 mm and 0.30 x 40/50 mm (XENO, HEGU Svenska AB, Landsbro, Sweden and in China Huatuo, Suzhou Medical Co Ltd, China) will be inserted to a depth of 15–40 mm in segmental acupuncture points located in abdominal and leg muscles, with innervations corresponding to the ovaries and the pancreas. Two sets of acupuncture points will be alternated every second treatment (Table 1). The first set of acupuncture points include points located in abdominal muscles: conception vessel (CV)4, CV12, stomach (ST)29 bilaterally, and in quadriceps muscle, ST32 and ST34, and in the muscles below the knee, spleen (SP)6, and ST36 bilaterally. In addition, needles are placed in the hand, large intestinal (LI)4, bilateral. All needles will be stimulated manually when inserted. CV4 and 12, ST29 bilateral, ST32 and ST34 bilateral will be connected to an electrical stimulator and stimulated with low-frequency EA of 2 Hz (Stimulators used in Sweden: Export Abteilung, Schwa-Medico GmbH, Wetzlarer Str. 41-43;35630 Ehringshausen and in China: Shanghai Huayi Electric Acupuncture Instrument: G6805-1A) for 30 min at each treatment. The intensity will be adjusted by the physiotherapist to produce local muscle contractions without pain or discomfort, and thereafter will the patient monitor the stimulation intensity. Six additional points are selected to strengthen the effect: LI4, ST36, and SP6, and they will be stimulated manually by rotating the needle to evoke needle sensation every 10 min.

The second set of acupuncture points include abdominal points: ST27 bilaterally (EA), CV6 to CV10 (EA); and leg points: SP10 to a non-acupuncture point located 6 cun proximal of patellas medial border (EA) are all connected to an electrical stimulator and stimulated as in the first set of acupuncture points. Six additional points; ST38, liver (LR)3 and pericardium (PC)6, all bilateral, are stimulated manually by rotating the needle to evoke needle sensation every 10 min.

Compliance: If a participant in the acupuncture group deviate considerably form the study protocol, the acupuncturists should inform the study coordinator. Any negative side effects during treatment are recorded.

Metformin: Oral metformin 500 mg three times daily, in total 1500 mg per day [23 24]. To reduce gastrointestinal side-effects of metformin, the dose will be slowly escalated starting with 500 mg daily during the first week, increasing to 500 mg twice per day during the second the week, and 500 mg three times daily, morning, lunch and dinner from the third week in total 16 weeks including the 3 weeks step-up phase (i.e. 4 months). Patients with negative effects can remain 500 mg during the remaining weeks.

Compliance: Empty bottles are handed over to the study coordinator after 16 weeks of treatment and number of tablets are counted. Also, once per month, the study coordinator call the participant and ask her to count the number of tablets there are left in the bottle.

Study Procedure

Screening: The study coordinator describes the study design in detail and written informed consent is collected. Of note, if a participant hesitates to go through tissue sampling as described below, this is not an exclusion criteria. All other outcome measures will be collected and are listed in Table 2.

In all participants, a comprehensive anamnestic interview will be conducted, including menstrual frequency, hirsutism - FG and acne determined by an affirmative answer to the question "Do you have excessive acne? yes or no", heredity, medication or other diseases. The physical examination including gynaecological examination is performed by transvaginal ultrasound (PCO morphology: yes or no). Body weight (kg) and body height (cm) are measured in an upright position with light clothing and no shoes. BMI is calculated as body weight (kg) divided by squared body height (m^2). Waist circumference is measured in centimetres at the midpoint between the iliac crest and lower rib margin at the end of expiration, while standing without clothing. Hip circumference is measured in centimetres at the widest point between waist and thighs. Waist-Hip-Ratio (WHR) is calculated as the ratio of waist and hip circumferences. Systolic (SBP) and diastolic blood pressure (DBP) is measured with a semiautomatic blood pressure monitor, and heart rate.

Each woman (PCOS and controls) is given seven questionnaires to be filled in and returned at next (baseline) visit. They are asked to start to register their bleeding periods from now until the end of study.

An appointment for body composition (lean and fat mass and bone mineral density) measure with dual energy x-ray absorptiometry (DXA) is given.

To enable measurements day 6-8 in the menstrual cycle, all women are given information on how to induce withdrawal bleeding with medroxyprogesterone acetate, 10 mg per day for 7 days (participants in Sweden) or dydrogesteron, 20 mg per day for 10 days (participants in China).

Baseline: The baseline visit takes place in the morning after an overnight fast on day 6 – 8 after induced withdrawal bleeding in all women (see above). The time point is selected as the

endometrial lining has to be thicker. The questionnaires are returned and checked. Missing information in the questionnaires is checked.

First, a gynaecological examination is performed by transvaginal ultrasound, measuring ovarian size in three dimensions, total antral follicle count (2-9 mm) and endometrial thickness (mm).

Second, if biopsies are taken, local anaesthesia will be placed and an endometrial biopsy is collected and snap frozen. Immediately after, local anaesthesia is placed close to the umbilicus and in the vastus lateralis muscle, fat and muscle biopsy are taken.

Third, a venflon will be placed and fasting blood samples will be drawn for serum and plasma analyses e.g. *genetic* (e.g. next generation sequencing, SNP, methylation), *metabolic* (e.g. lipids, adipokines, inflammatory markers) and *endocrine* (e.g. sex steroids, gonadotropins, growth factors) measures.

Fourth, an oral glucose tolerance test (OGTT) with 75 g glucose will be performed. Blood samples is collected to measure plasma glucose and serum insulin at 0, 30, 60, and 120 min during the OGTT.

At the baseline visit, after OGTT, all participants will receive lifestyle advice by the study coordinator. Patient will be told to register daily number of steps and will receive a step-counter and asked to register menstrual bleeding. If allocated to the electroacupuncture group, time will be booked and treatment started within one week. If randomized to the metformin group, the study drug will be administered and the treatment started the next day. The lifestyle management only group are given appointments for repeated measurements after 4 months and follow-up 4 months later.

Women with PCOS who are randomized are informed that they should use contraception that are non-hormonal.

Follow-up 4 months after last treatment: All baseline measures are repeated after 4 months of treatment and at follow-up 4 months after last treatment.

Randomisation and treatment allocation

The randomization will employ a minimization algorithm to balance across the following factors: Age and BMI and are separated by centre. Each study site (Stockholm and Beijing) use the same randomization and electronic case report form (eCRF). A web-based

randomization program (https://data.dynareg.se/pia2/Default.aspx) has been generated to ensure allocation concealment. The study coordinators log on the web-based system to randomize eligible patients. All women who enter the study will be logged and given a unique study number. Blinding or masking of the intervention will not be possible because of the nature of the intervention. Importantly, however, the assessor will be blinded to the patients' group assignment.

Outcome Measurements

Outcome measures will be collected at:

1. Baseline
2. After 4 months of intervention
3. Follow-up 4 months after last treatment

Primary Outcome

At baseline in cases versus controls and in women with PCOS changes from baseline to after 4 months of treatment in

- HOMA-IR [fasting insulin (μU/mL) × fasting glucose (mmol/L)] / 22.5)], and
- HbA1c

comparing 1) acupuncture + lifestyle management and 2) metformin + lifestyle management and 3) lifestyle management only.

Secondary Outcome

At baseline in cases versus controls and in women with PCOS changes from baseline to after 4 months of treatment and from baseline to the 4-month follow-up between 1) acupuncture + lifestyle management, and 2) metformin + lifestyle management, compared to 3) lifestyle management only, in the following variables:

- *Detailed characterisation of body composition*: In addition to weight, height and waist circumference, all women with be examined by DXA to measure lean and fat mass and bone mineral density using a Lunar Prodigy Advance whole body scanner (GE Medical Systems) [25].
- *Metabolic measures:* Insulin response to glucose during the OGTT (AUC using the trapezoidal rule), and direct analyses of fasting blood samples of insulin an glucose to

enable calculation of HOMA-B /islet β-cell function [20 × fasting insulin (mU/mL) / (fasting plasma glucose (mmol/L) − 3.5)] [26]. Further, fasting blood samples are collected and saved for later analyses of e.g. C-peptide and calculation of C-peptide index [Fasting C-peptide (nmol/L)/ f-glucose (mmol/L) x 100] [27], for analyses of adipokines, inflammatory markers, non-esterified fatty acids (NEFA), total cholesterol, triglycerides, high density lipoprotein (HDL), low density lipoprotein (LDL), high sensitive CRP, catecholamine's; norepinephrine (NE), adrenalin (A) and dopamine (DA).

- *Endocrine measures*: Menstrual frequency: Participants will be asked to note date of menstruation which will be reported to the study coordinator once per week by text message and every 4th week by phone. Ovarian morphology antral follicle count and ovarian volume. Blood samples will be drawn for analyses of sex steroids by the validated gas- and liquid chromatography/tandem mass spectroscopy technique, as well as sex hormone binding globulin (SHBG), luteinizing hormone (LH), follicle stimulating hormone (FSH), antimüllerian hormone (AMH), prolactin, thyroid stimulating hormone (TSH) and free thyroxine (T4).
- *Tissue and whole blood collection:* Whole blood will be collected for DNA and microRNA analyses. Endometrial, fat and skeletal muscle tissue biopsies will be collected at baseline (cases and controls), after 4 months of treatment and at follow-up 4 months after treatment in women with PCOS, snap frozen in liquid nitrogen within 30 s and stored at -80°C for later analyzes. Fat cells will be isolated for determination of adipocyte size and distribution. Part of tissue biopsies will also be isolated for *in vitro* experiments.[28] Deep RNA, microRNA and/or bisulfite sequencing will be performed with the latest available technology.
- *Health related quality of life:* Will be determined by quality of life by EuroQol-5 dimension (EQ-5D),[29 30] short form-36 (SF36),[31 32] and polycystic ovary syndrome questionnaire (PCOSQ).[33 34]
- *Symptoms of anxiety and depression* will be assessed by the self-reported version of the Comprehensive Psychopathological Rating Scale for Affective Syndromes (CPRS-S-A)[35] to assess psychiatric symptoms within a time frame of the last 3 days in Sweden. For the purpose of this study, two scales will be extracted from the CPRS-S-A,[35] the Brief Scale for Anxiety (BSA-S)[36] and the Montgomery Åsberg Depression Rating Scale (MADRS-S).[37-39] In China will the Zung symptom depressions score (SDS) and Zung symptom

anxiety score (SAS) be used [40 41]. Depression symptoms of potential clinical relevance is for MADR-S ≥11 and for Zung SDS ≥ 0.5 (Depressive index), Anxiety symptoms of potential clinical relevance is for BSA-S ≥11 and for Zung SAS ≥50 (Standard total score),

- *Physical Activity:* International Physical Activity Questionnaire (IPAQ) long and short version will be used to assess physical activity.[42 43] In addition, one text message per week will be sent to the participants by the study coordinators asking of number of steps the last week when asking for menstrual bleeding (date).
- *Eating questionnaire and eating pattern:* Only assessed at baseline using the self-reported version of the Three-Factor Eating Questionnaire (TFEQ-R21),[44 45] and Questionnaire of Eating and Weight Patterns-Revised (QEWP-R) to measure eating behaviour (Sweden only).[46]
- *Side-effects and adverse events* will be continuously and equally recorded in each study arm. One text message per week will be sent to all participants by the study coordinators in which they are asked to report (in addition to number of steps) any side effects or adverse events. All participants will receive a phone call every 4th week by the study coordinator and will be asked about side effects or adverse events.

Statistical analysis

Sample size and power calculations

Samples size calculation derives from our pilot study demonstrating that 5 weeks of treatment decreases HOMA-IR by meanΔ –0.62, standard deviation (SD) 1.21 and HbA1c by meanΔ –1.30, SD 1.40.[15] Here we expect a decrease in HOMA-IR from baseline to after 4 months of treatment with metformin or acupuncture with an anticipated mean difference of 0.43 and a standard deviation of 1 compared to lifestyle management alone. With a target power of at least 80% and 5% significance level, we need to recruit 84 women per group. We plan to recruit 101 women per group estimating 20% dropout rate. If statistical power is calculated based on HbA1c, with an anticipated mean difference of 0.86 and a standard deviation of 1.4 compared to lifestyle management alone, and with a target power of at least 80% and 5% significance level, we would need to recruit 23 women (28 if estimating a 20% dropout) per group.

As this is a comparative effectiveness trial we have decided to have two primary outcome variables and accordingly we have calculated the samples size for the different outcomes. The primary end-point HOMA-IR requires the highest number of participants. When the intended number of participants for Hba1c have been reached, an interim analyses will be performed.

Further, for the mechanistic studies, we estimate that successful tissue samples will be recruited from a minimum 20 participants in each group in Sweden and China respectively, giving a strong power to detect differences.

Minimizing sources of bias

Blinding is not possible given the nature of the intervention. We do not feel it is necessary or ethical to perform sham acupuncture and are confident that the primary outcome is unlikely to be affected by observer bias.

Type of analyses

The statistical analyses will be performed by qualified statisticians and biostatisticians. The data in the RCT will be analysed according to the intent-to-treat principle to investigate differences between the groups.

Interim Analysis: After 114 subjects (number based on samples size calculation of Hba1c) have finalized the 16 weeks of treatment with follow-up, an interim analysis will be conducted. The interim data will be used to check assumptions in the sample size calculation for both co-primary variables and samples size will be recalculated based on observed data. The stop criterion are meant for both co-primary and covers two group comparisons. The Haybittle-Peto approach is used and the trial will be ended using symmetric stopping boundaries at $P < 0.001$. Bonferroni will be used for correction for multiple testing. The final analyses is evaluated at the significance level $P < 0.05$, with posthoc corrections as given below.

Clinical outcome measures: Continuous variables will be presented as means ± standard deviations and categorical variables as medians with interquartile ranges. Between group comparisons will be carried out with changes from baseline to after treatment and from baseline to follow-up by ANOVA followed by Dunnet post-hoc test for continuous and Kruskal-Wallis followed by Mann Whitney U-test or by χ^2 tests for categorical variables.

In the case-control part of the study the Student t-test will be used for continuous variables and Mann Whitney U-test or χ^2 tests for categorical variables and logistic regression when needed.

All statistical analyses of the data will be performed using the SPSS program version 23.0 or higher (SPSS Inc., Chicago, IL, USA), and a *P*-value < 0.05 will be considered statistically significant. All tests are two-sided and adjustments for multiple comparisons will be performed.

Expression and methylation data: These analyses will be adjusted to the technique used. In brief, raw data will be checked and processed and a quality control report will be completed. Different analysis pipelines for traceability and track-ability will be performed. Then extended data analyses, including functional analysis, GeneOntologies, Biological Pathways, Principle Component Analysis (PCA)-analysis, Clustering, Visualizations and mapping against a reference genome, will be performed, and data will be submitted to repositories (i.e. the Array Express: www.ebi.ac.uk/arrayexpress).

Group comparison will be carried out with changes from baseline to after treatment and from baseline to follow-up by Kruskal-Wallis followed by Mann Whitney U-test for expression analyses. In the case-control part of the study, Mann Whitney U-test will be used for expression analyses. False discovery rate (FDR) will be used to correct for multiple testing in the analyses of gene and methylation arrays.

Ethics and Dissemination

The study is performed according to good clinical practice and conducted in accordance with the Declaration of Helsinki. The study has been approved by the Regional Ethical Review Board of Stockholm, Sweden Dnr: 2015/1656-31/2 and by the Regional Ethical Review Board of Peking University Third Hospital, China Dnr: 2016-212-02. In addition, the Medical Products Agency have approved the study: EudraCT: 2015-004250-18 and the trial is registered at Clinicaltrials.gov: NCT02647827. Reporting of the study results will follow the 2010 revised CONSORT statement and STRICTA.[47 48] Primary outcome data the RCT will be published in a relevant journal together with supporting secondary outcome measurements. Further, secondary outcome measurements will be published in separate papers as well as case-control data.

The relevance of this study is that it has potential to uncover new knowledge in the pathophysiology of the disorder and result an additional treatment strategy for insulin resistant in women with PCOS and related diseases, including obesity, insulin resistance, and T2D. Thus, it may have an impact on both genders and does not apply only to women with PCOS.

Trial status

The study was conceived and designed during 2015. The first participant was recruited and randomized in February 2016 in Sweden and September 2016 in China. We anticipate that all participants are recruited by the end of 2019 with follow-up done during 2020.

Funding Statement

The work is supported by the Swedish Medical Research Council (Project No. 2014-2775); Adlerbert Research Foundation; Novo Nordisk Foundation (NNF17OC0026724); Strategic Research Programme (SRP) in Diabetes at Karolinska Institutet; Swedish federal government under the Regional agreement on medical training and clinical research (ALF) between Stockholm County Council and Karolinska Institutet (all ESV). In China, this study is supported by the National Key Research and Development Program (2016YFC100021), National Key Technology R&D Program (2015BAI13B06, 2014BAI05B04), National Natural Science Foundation of China (81603446), Beijing Municipal Natural Science Foundation (7174363).The funders have had no role in study design, and will not have any role in data collection and analysis, decision to publish, or preparation of the manuscripts.

Competing interest

The authors declare that they have no competing interests.

Author contribution

The authors declare that they have no competing interests.

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Table 1: Acupuncture points, stimulation, localization, tissue in which needles are inserted, and innervation areas. The two sets will be alternated for every other treatment.

Acupuncture point	Stimulation	Localization	Muscle	Muscle innervation
Set 1				
CV4, *Guan Yuan*	EA	3 cun caudal to the umbilicus	Fibrous tissue, linea alba	L1
CV12, *Zhongwan*	EA	On the midline, 4 cun superior to the umbilicus	Fibrous tissue, linea alba	Th7–8
ST29 Bilateral, *Guilai*	EA	1 cun cranial to the pubic bone and 2 cun lateral of the midline	M. rectus abdominis	Th6–12
ST34 Bilateral, *Futu*	EA	2 cun above the superior lateral border of the patella on the line connecting the anterior superior iliac spine found	M. quadriceps femoris	femoral nerve
ST32 Bilateral, *Liangqiu*	EA	6 cun above the superior lateral border of the patella on the line connecting the anterior superior iliac spine found	M. quadriceps femoris	femoral nerve
SP6 Bilateral, *Sanyinjiao*	DeQi, four times	3 cun proximal to the medial malleolus	Mm. flexor digitorum longus, tibialis posterior	L4–5, S1–2
ST36 Bilateral, *Zusanli*	DeQi, four times	On the anterior lateral side of the leg, 3 cun below *Dubi* (ST35), one finger width (middle finger) from the anterior crest of the tibia	Musculi tibialis anterior	L4–5, S1
LI4 Bilateral, *Hegu*	DeQi, four times	On the highest point at m. interosseus dorsalis	Mm. interosseus dorsalis I, lumbricalis II, adductor pollicis	C8, Th1

Set 2				
CV6, *Qihai*	EA	1.5 cun caudal to the umbilicus	Fibrous tissue, linea alba	Th11
CV10, *Xiawan*	EA	2 cun cranial to the umbilicus	Fibrous tissue, linea alba	Th8
ST27 Bilateral, *Daju*	EA	3 cun cranial to the pubic bone and 2 cun lateral to the midline	M. rectus abdominis	Th6–12
Extra meridian point Bilateral	EA	6 cun above the patella in line with SP10	M. quadriceps femoris	L2–L4
SP10 Bilateral, *Xuehai*	EA	With the knee flexed, on the medial side of the thigh 2 cun above the superior medial corner of the patella on the prominence of the medial head of the quadriceps muscle of the thigh	M. quadriceps femoris	L2–L4
ST38 Bilateral, *Sanyinjiao*	DeQi four times	Between lateral malleolus and knee joint, 1 finger from tibiae crist	Musculi tibialis anterior	L4–5, S1
LR3 Bilateral, *Taichong*	DeQi four times	Between metatarsal I & II, just distal to the caput	M. Interosseus dorsalis I	S2–3
PC6 Bilateral, *Neiguan*	DeQi four times	2 cun proximal to the processus styloideus radii, between the tendons of the palmaris longus and the flexor carpi radialis	M. flexor digitorum superficialis	C8, Th1

C: Cervical vertebra; CV: Conception vessel; L: Lumbar vertebra; LI: Large intestine; LR: Liver; PC: Pericardium; S: Sacral vertebra; SP: Spleen; ST: Stomach; Th: Thoracic vertebra.

Table 2. Overview of the study visits.

	Screening visit	**Base line visit**	**Month**				**Follow-up after 4 months of treatment**	**Follow-up 4 months after last treatment**
			1st	2nd	3rd	4th		
Anthropometry: Body composition (weight, height, waist circumference, hip circumference), FG/acne, blood pressure	X						X	X
Menstrual cycle diary	X	X	X	X	X	X	X	X
Questionnaires: EQ-5D, SF36, PCOSQ, CPRS-SA*, Zung SAS#, Zung SDS#, IPAQ, TFEQ-R21, QEWP-R*	X						X	X
Transvaginal ultrasound		X					X	X
Metabolic measures: Fasting blood samples for glucose, insulin, HbA1c, c-peptide, OGTT. Adipokines, lipid profile (LDL, HDL, NEFA) and inflammatory markers		X					X	X
DXA		X					X	X
Endocrine measures: Fasting blood samples for sex steroids, SHBG, LH, FSH, AMH, prolactin, TSH, T4		X					X	X
Tissue and whole blood collection		X					X	X

Antimüllerian hormone (AMH); dual energy x-ray absorptiometry (DXA); EuroQol-5 dimension (EQ-5D); Ferriman–Gallwey score (FG); follicle stimulating hormone (FSH); high density lipoprotein (HDL); hsCRP, International Physical Activity Questionnaire (IPAQ); low density lipoprotein (LDL), luteinizing hormone (LH); non-esterified fatty acids (NEFA); norepinephinre (NE), oral glucose tolerance test (OGTT); polycystic ovary syndrome questionnaire (PCOSQ); Questionnaire of Eating and Weight Patterns-Revised (QEWP-R); sex hormone binding globulin

(SHBG); short form-36 (SF36); Three-Factor Eating Questionnaire (TFEQ-R21); thyroid stimulating hormone (TSH); thyroxine (T4); Zung Self-Rating Anxiety Scale (Zung SAS); Zung Self-Rating Depression Scale (Zung SDS).

* only in Sweden and # only in China.

BMJ Open

Acupuncture or metformin to improve insulin resistance in women with polycystic ovary syndrome: Study protocol of a combined multinational cross sectional case-control and a randomized controlled trial

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2018-024733.R1
Article Type:	Protocol
Date Submitted by the Author:	18-Aug-2018
Complete List of Authors:	Stener-Victorin, Elisabet; Karolinska Institutet, Department of Physiology and Pharmacology Zhang, Haolin; Peking University Third Hospital, Department of Traditional Chinese Medicine (TCM) Rong, LI; Peking University Third Hospital, OB & GYN Friden, Cecilia; Karolinska Institutet, 5. Department of Neurobiology, Care Sciences and Society, Division of Physiotherapy Li, Dong; Peking University Third Hospital, Department of Traditional Chinese Medicine (TCM) Wang, Wei; Peking University Third Hospital, 4. Department of Obstetrics and Gynecology, Peking University Third Hospital, Beijing, China Wang, Haining ; Peking University Third Hospital, Department of Endocrinology and Metabolism, Peking University Third Hospital, Beijing, China Chang , Cuiqing; Peking University Third Hospital, Institute of Sports Medicine Li , Shi; Peking University Third Hospital, 3. Center for Reproductive Medicine, Department of Obstetrics and Gynecolog Huo , ZeJun ; Peking University Third Hospital, Research Center of Clinical Epidemiology Zhang, Hua; Peking University Third Hospital, Research Center of Clinical Epidemiology Ji, Xiaolan ; Peking University Third Hospital, Department of Traditional Chinese Medicine (TCM) Linden-Hirschberg, Angelica; Karolinska Institutet, Department of Obstetrics and Gynecology Jie, Qiao; Peking University Third Hospital, Center for Reproductive Medicine, Department of Obstetrics and Gynecology andDepartment of Obstetrics and Gynecology, Peking University Third Hospital, Beijing, China
Primary Subject Heading:	Diabetes and endocrinology
Secondary Subject Heading:	Complementary medicine
Keywords:	glucose homeostasis, insulin resistance, acupuncture, life style, polycystic ovary syndrome

Acupuncture or metformin to improve insulin resistance in women with polycystic ovary syndrome: Study protocol of a combined multinational cross sectional case-control and a randomized controlled trial

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Short title: Acupuncture or metformin for insulin resistance in PCOS

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Author Contributions

ESV, HZ, RL, ALH and JQ conceived and designed the study. ESV and JQ sought funding and ethical approval. CF, DL, WW, HW, CC, SL, ZH, HZ, XJ and ALH recruited and screened subjects, coordinated and carried out acupuncture treatment. ESV drafted the manuscript. All authors read and approved the final manuscript.

Abstract

Introduction: Polycystic ovary syndrome (PCOS) is linked to hyperinsulinemia and insulin resistance with dysfunctional glucose metabolism. Pilot studies suggests that acupuncture treatment with combined manual and low-frequency electrical stimulation (electroacupuncture, EA) of the needles decrease circulating glycated hemoglobulin (HbA1c) and homeostatic model assessment-insulin resistance (HOMA-IR). Therefore, we here aim to investigate if acupuncture treatment or metformin together with life style or life style management alone improve insulin sensitivity and related symptoms in overweight/obese women with PCOS.

Methods and analysis: This is a two centre multinational (Sweden and China), cross-sectional case-control study combined with an open-labelled randomized controlled trial (RCT). Participants are randomized to one of three groups: 1) EA 2-3 times/week during 4 months + lifestyle management; 2) metformin, 500 mg, three/day during 4 months + lifestyle management; or 3) life style management alone. The primary outcome measure in the RCT is changes in Hba1C. A total of 123 obese overweight women with PCOS will be enrolled and randomized into one of the three groups with a target power of at least 80% and 5% significance level based on two-sided tests.

Ethics and dissemination: The study has been approved by the Regional Ethical Review Board of Stockholm and of Peking University Third Hospital, China. Primary outcome data of the RCT will be published in a relevant journal together with supporting secondary outcome measurements. Further, outcome measurements will be published in separate papers as well as case-control data.

Expected results: We anticipate that EA and metformin, both with lifestyle management are equally effective and superior to lifestyle management alone for improvement of glycemic control.

Trial registration: Clinicaltrials.gov: NCT02647827 and EudraCT: 2015-004250-18.

Key words: glucose homeostasis; insulin resistance; metformin; electroacupuncture; lifestyle

Strength and Limitations

1. A strength of this trial is that all patients will benefit from receiving treatment, all of which alone and/or in combination may offer an increased chance for improved metabolic function and reproductive health.

2. It has the potential to gain deeper insight into the pathophysiology of polycystic ovary syndrome (PCOS), and to uncover new knowledge for treatment of insulin resistant in related diseases, including obesity, insulin resistance, and type 2 diabetes (T2D).

3. The results from the present study have the potential to immediately be implemented into the healthcare system since it has previously been shown to be cost-effective and to have few negative side effects.

4. A potential limitation is that metformin might cause side-effects such as diarrhoea, nausea/vomiting, flatulence, asthenia, indigestion, abdominal discomfort and headache and acupuncture local skin irritation, discomfort, and vasovagal reactions during the procedure.

Background

Polycystic Ovary Syndrome (PCOS) is the most common endocrine and metabolic disorder in women of reproductive age and is characterized by anovulation, hyperandrogenism and metabolic dysfunction.[1] Women with PCOS have a 3 to 7-fold increased risk of developing type 2 diabetes (T2D), and with younger onset, PCOS increases cardiovascular risk factors[1-6]. Independent of body weight, insulin sensitivity is ~40% lower in women with PCOS than in healthy women, and impaired glucose regulation, insulin resistance and reduced insulin responsiveness have been attributed to defects in insulin signalling in adipocytes and skeletal muscle.[7 8] Of note, obesity aggravates all symptoms related to PCOS.

Despite detrimental impact on women's health, the aetiology of PCOS is not well understood. Genetic, epigenetic, and environmental factors have all been implicated in its development. Emerging evidence suggests that PCOS originates, at least in part, in fetal life,[9 10] and elevated maternal androgens have been implicated to play a role, however the mechanisms are largely unknown.[11 12] Of interest is that we have found that women with PCOS have multiple transcriptional and epigenetic changes in adipose tissue that are relevant for development of the disease.[13] Further, twin studies suggest that genetic influences explain >70% of PCOS pathogenesis.[14] However, whether genetic or epigenetic alterations in target tissues e.g. adipose tissue, skeletal muscle and endometrium contribute to development of metabolic disease requires further investigation.

Women with PCOS require long-term individualized treatment programs. Pharmacological treatments, including the glucose reducing drug metformin, have limitations related to adverse effects and patient compliance. Therefore, there is a need for inexpensive and easily administered treatments with few negative side-effects. Lifestyle management is the first line treatment eventually with addition of metformin for improving whole body glucose homeostasis and preventing type 2 diabetes (T2D).[1 15-17] Interestingly, five weeks of acupuncture with combined manual and low-frequency electrical stimulation has in a pilot study been shown to improve whole body glucose homeostasis in insulin resistant women with PCOS.[18] The pilot study was an uncontrolled trial and it is therefore of importance to compare the effect of acupuncture with first line treatment to investigate the effectiveness.

Whilst pharmacological treatment strategies have shown efficacy, importantly, there is a need for Comparative Effectiveness Research (CER) to strengthen the evidence base for clinical and policy decision-making [19]. Therefore we aim to compare the effect of pharmacological first-line treatment, metformin, with a non-pharmacological treatment strategy, acupuncture

(both together with lifestyle management), with lifestyle management for improvement and prevention of metabolic dysfunction and related symptoms in insulin resistant women with PCOS.

Our main hypothesis is that acupuncture and metformin (both treatments combined with lifestyle management) are superior to lifestyle management alone in improving whole body glucose regulation in insulin resistant women with PCOS. Secondary hypotheses are that these treatments have the potential to improve metabolic- and endocrine measures, quality of life and symptom of anxiety and depression, and to restore epigenetic and molecular alterations in target tissues (endometrial-, adipose-, and skeletal muscle tissue) and thus have the potential to improve and potentially prevent the development of metabolic alterations including T2D.

Thus, the purpose of this study is twofold, first we aim to gain deeper insight into the pathophysiology of PCOS in a cross sectional case-control study by comparing women with PCOS with women without PCOS matched for age, weight and BMI, and secondly we aim to perform a prospective RCT of women with PCOS.

Study design

This is a two centre multinational prospective trial with a cross-sectional case-control part and an open-labelled RCT with a comparative effectiveness design. The interventions to be tested are 1) Electroacupuncture during 4 months + lifestyle management; 2) Metformin during 4 months + lifestyle management; or 3) Lifestyle management alone which will be available for participants in all three groups. Participants will be enrolled at Karolinska Institutet and Karolinska University Hospital, Stockholm, Sweden and at Peking University Hospital, Beijing China respectively.

Randomisation and treatment allocation

The randomization is stratified across the factors age and BMI and is also separated by study site with a balanced allocation ratios 1:1:1. Randomization is performed in blocks with a variable block size between 3 and 15; e.g. First there is a block of 12, when it is full it is followed by a block of 9 and thereafter a block of 3. The order of the block sizes are unknown to the participating study sites and also differs among the strata's.
Each study site (Stockholm and Beijing) use the same randomization and electronic case report form (eCRF). A web-based randomization program (https://data.dynareg.se/pia2/Default.aspx) has been generated to ensure allocation

concealment. The study coordinators log on the web-based system to randomize eligible patients. All women who enter the study will be logged and given a unique study number. Blinding or masking of the intervention will not be possible because of the nature of the intervention. Importantly, however, the assessor will be blinded to the patients' group assignment.

Patient and Public Involvement

Patients and or public was not involved in the design of this study.

Study Objectives

Primary Objective

1. To determine the clinical effectiveness of 4 months of 1) electroacupuncture + lifestyle management and 2) metformin + lifestyle management, compared to 3) lifestyle management only for improvement of glucose regulation assessed by Hba1c levels.

Secondary Objectives

1. To evaluate changes in secondary metabolic measures including the insulin response to glucose assessed by calculating the area under the curve ($AUC_{insulin}$) during the oral glucose tolerance test (OGTT), fasting insulin, glucose, calculation of homeostatic model assessment (HOMA)-IR and-HOMA-B (*i.e.* the Islet β-cell function) and the assessment of e.g. adipokines, lipid profile, body size and proportions and body fat distribution.
2. To determine changes in gene expression and DNA methylation profiles related to insulin sensitivity in fat, muscle and endometrial tissue biopsies, and biomarkers in whole blood.
3. To evaluate endocrine measures including menstrual pattern and ovulation frequency, circulating hormones (e.g. sex steroids, AMH, gonadotropins).
4. To determine changes in women's health related quality of life (HRQoL), symptoms of anxiety and depression, dieting and eating patterns, and negative side-effects.

Outcome Measurements

Outcome measures will be collected at:

1. Baseline
2. After 4 months of intervention

3. Follow-up 4 months after last treatment

Primary Outcome

Changes from baseline to after 4 months of treatment in HbA1c comparing 1) acupuncture + lifestyle management and 2) metformin + lifestyle management, respectively with 3) lifestyle management only. In the cross sectional study, difference in HbA1c between cases and controls.

Secondary Outcome

Changes from baseline to after 4 months of treatment and from baseline to the 4-month follow-up comparing 1) acupuncture + lifestyle management, and 2) metformin + lifestyle management, respectively, with 3) lifestyle management only, and in the cross sectional study, difference between cases and controls in the following variables:

- *Body composition*: In addition to weight, height and waist circumference, women will be examined by DXA to measure lean and fat mass and bone mineral density using a Lunar Prodigy Advance whole body scanner (GE Medical Systems) [20].
- *Metabolic measures:* Insulin response to glucose during the OGTT (AUC using the trapezoidal rule), and direct analyses of fasting blood samples of insulin an glucose to enable calculation of HOMA-IR [fasting insulin (μU/mL) × fasting glucose (mmol/L)] / 22.5)], and HOMA-B /islet β-cell function [20 × fasting insulin (mU/mL) / (fasting plasma glucose (mmol/L) − 3.5)] [21]. Further, fasting blood samples are collected and saved for later analyses of e.g. C-peptide and calculation of C-peptide index [Fasting C-peptide (nmol/L)/ f-glucose (mmol/L) x 100] [22], for analyses of adipokines, inflammatory markers, non-esterified fatty acids (NEFA), total cholesterol, triglycerides, high density lipoprotein (HDL), low density lipoprotein (LDL), high sensitive CRP, catecholamine's and metabolites analysed on a split-fraction HPLC-ED system [23].
- *Endocrine measures*: Menstrual frequency: Participants will be asked to note date of menstruation which will be reported to the study coordinator once per week by text message and every 4th week by phone. Ovarian morphology antral follicle count and ovarian volume. Blood samples will be drawn for analyses of sex steroids by the validated gas- and liquid chromatography/tandem mass spectroscopy technique, as well as sex hormone binding globulin (SHBG), luteinizing hormone (LH), follicle stimulating hormone (FSH), antimüllerian hormone (AMH), prolactin, thyroid stimulating hormone (TSH) and free thyroxine (T4).

- *Tissue and whole blood collection:* Whole blood will be collected for DNA and microRNA analyses. Endometrial, fat and skeletal muscle tissue biopsies will be collected at baseline (cases and controls), after 4 months of treatment and at follow-up 4 months after treatment in women with PCOS, snap frozen in liquid nitrogen within 30 s and stored at -80°C for later analyzes. Fat cells will be isolated for determination of adipocyte size and distribution. Part of tissue biopsies will also be isolated for *in vitro* experiments.[24] Deep RNA, microRNA and/or bisulfite sequencing will be performed with the latest available technology.
- *Health related quality of life:* Will be determined by quality of life by EuroQol-5 dimension (EQ-5D),[25 26] short form-36 (SF36),[27 28] and polycystic ovary syndrome questionnaire (PCOSQ).[29 30]
- *Symptoms of anxiety and depression* will be assessed by the self-reported version of the Comprehensive Psychopathological Rating Scale for Affective Syndromes (CPRS-S-A)[31] to assess psychiatric symptoms within a time frame of the last 3 days in Sweden. For the purpose of this study, two scales will be extracted from the CPRS-S-A,[31] the Brief Scale for Anxiety (BSA-S)[32] and the Montgomery Åsberg Depression Rating Scale (MADRS-S).[33-35] In China will the Zung symptom depressions score (SDS) and Zung symptom anxiety score (SAS) be used [36 37]. Depression symptoms of potential clinical relevance is for MADR-S ≥11 and for Zung SDS ≥ 0.5 (Depressive index), Anxiety symptoms of potential clinical relevance is for BSA-S ≥11 and for Zung SAS ≥50 (Standard total score),
- *Physical Activity:* International Physical Activity Questionnaire (IPAQ) will be used to assess degree of physical activity.[38 39] In addition, one text message per week will be sent to the participants by the study coordinators asking of number of steps the last week when asking for menstrual bleeding (date).
- *Eating questionnaire and eating pattern:* Only assessed at baseline using the self-reported version of the Three-Factor Eating Questionnaire (TFEQ-R21),[40 41] and Questionnaire of Eating and Weight Patterns-Revised (QEWP-R) to measure eating behaviour (Sweden only).[42]
- *Side-effects and adverse events* will be continuously and equally recorded in each study arm. One text message per week will be sent to all participants by the study coordinators in which they are asked to report (in addition to number of steps) any side effects or

adverse events. All participants will receive a phone call every 4th week by the study coordinator and will be asked about side effects or adverse events.

Participants

Eligible women will be identified by their clinician, or by local newspaper advertisements, and invited to participate in the trial. Each participant will be given written and oral information and asked for her signed informed consent to be randomized and followed-up by research staff. The cross sectional case-control part of the study equals baseline measurements for women with PCOS and controls. If a patient do not adhere to the frequent treatment this will be clearly tracked as the treatment may still be effective.

Inclusion criteria – women with PCOS

1. Age ≥18 to ≤40 years
2. Body mass index (BMI) ≥25 to ≤40 given that 95% of all women with PCOS with a BMI ≥25 are insulin resistant.[43 44]
3. PCOS diagnosis according to Rotterdam criteria 2003 [45], with at least two of the following three symptoms: Clinical and/or biochemical signs of hyperandrogenism (hirsutism or acne); oligo/amenorrhea; and/or polycystic ovaries (PCOS). Biochemical hyperandrogenism is defined by total testosterone >1.2 nmol/L or a free androgen index (FAI)>5.[46] Hirsutism is defined as a self-reported Ferriman-Gallwey (FG) score ≥8 (≥5 Asian).[47 48] Acne is defined by a positive response to the question *Do you have acne?* Oligomenorrhea is defined as an intermenstrual interval >35 days and <8 menstrual bleedings in the past year. Amenorrhea as <3 cycles per year. PCO is defined by transvaginal ultrasound with ≥12 follicles 2–9 mm and/or ovarian volume ≥10 ml in one or both ovaries.
4. Willing to sign the consent form.

Inclusion criteria – controls

Controls should have BMI ≥25 to ≤40, regular cycles with 28 days ± 2 days, and no signs of hyperandrogenism. They are excluded if they have menstrual irregularities, signs of hyperandrogenism (FG >4), or evidence of PCO morphology on ultrasound.

Exclusion criteria for all women

1. Exclusion of other endocrine disorders such as non-classic congenital adrenal hyperplasia (17-hydroxyprogesterone < 3nmol/L), androgen secreting tumors or suspected Cushing's syndrome.
2. Having known renal disease (creatinine clearance < 60 mL/min), hepatic insufficiency, autoimmune disorders or cancer.
3. Any acute condition with potential to alter renal function or cause tissue hypoxia.
4. Type I diabetes.
5. Pharmacological treatment (cortizon, antidepressant, other antidiabetic treatment such as insulin and acarbose, hormonal contraceptives, hormonal ovulation induction or other drugs judged by discretion of investigator) within 12 weeks. Depo Provera or similar within 6 months.
6. Hypersensitivity to metformin hydrochloride or to any of the excipients.
7. Blood pressure >160 / 100 mmHg
8. Pregnancy or breastfeeding the last 6 months
9. Acupuncture the last 2 months
10. Daily smoking and alcoholic intake
11. Language barrier or disabled person with reduced ability to understand the information given.

In total 50 controls will be matched at baseline (age, weight and BMI) to women with PCOS. Controls will undergo screening and baseline visit, but will not be randomized to any treatment.

Interventions

Participants fulfilling the inclusion criteria will be randomized to one of three groups after baseline measurements:

1. Electroacupuncture 2-3 times/week during 4 months + lifestyle management
2. Metformin, 500 mg, three times/day during 4 months + lifestyle management
3. Lifestyle management alone which will be available for participants in all three groups.

Lifestyle management: All women will receive lifestyle management instructions at the baseline visit, before randomization. The lifestyle management involves one initial

counselling session in connection with the baseline visit, which includes information about the importance of weight management, healthy diet and physical activity. Focus will be on the importance of physical activity. Each participant will receive a book with lifestyle advice about weight reduction, maintenance and physical activity following WHO recommendations. All participants will receive a text message once weekly to respond number of step collected by their smart phone or step counter during the last week and if they have had any menstrual bleedings. Once every fourth week, study coordinator will call to the participant and ask about number of step last week, menstruation and compliance and side-effects.

Electroacupuncture: Women randomized to receive acupuncture will start their treatment within one week after baseline measurements. The rationale of the acupuncture protocol is based on Western Medical Acupuncture theories. We will use a fixed acupuncture protocol following the two pilot studies: ClinicalTrial.gov NCT01457209 and NCT02026323 with two modifications. First, the treatment period will be 16 weeks (i.e. 4 months) compared to 5 weeks and 6 months in the previous pilot studies. Second, the treatment frequency will be 2 to 3 times per week during 16 weeks, i.e. in total 32 to 48 acupuncture treatments over 16 weeks. The rational for these changes is that the procedure is time-consuming for the patients and this will increase the feasibility and likely reduce the number of dropouts. Acupuncture treatment will be given by registered physiotherapists or medical doctors educated in theoretical and practical acupuncture and trained to follow the fixed protocol.
Disposable, single-use, sterilized CE marked needles made of stainless steel, 0.25 x 30 mm and 0.30 x 40/50 mm (XENO, HEGU Svenska AB, Landsbro, Sweden and in China Huatuo, Suzhou Medical Co Ltd, China) will be inserted to a depth of 15–40 mm in segmental acupuncture points located in abdominal and leg muscles, with innervations corresponding to the ovaries and the pancreas. Two sets of acupuncture points will be alternated every second treatment (Table 1). The first set of acupuncture points include points located in abdominal muscles: conception vessel (CV)4, CV12, stomach (ST)29 bilaterally, and in quadriceps muscle, ST32 and ST34, and in the muscles below the knee, spleen (SP)6, and ST36 bilaterally. In addition, needles are placed in the hand, large intestinal (LI)4, bilateral. All needles will be stimulated manually when inserted. CV4 and 12, ST29 bilateral, ST32 and ST34 bilateral will be connected to an electrical stimulator and stimulated with low-frequency EA of 2 Hz (Stimulators used in Sweden: Export Abteilung, Schwa-Medico GmbH, Wetzlarer Str. 41-43;35630 Ehringshausen and in China: Shanghai Huayi Electric

Acupuncture Instrument: G6805-1A) for 30 min at each treatment. The intensity will be adjusted by the physiotherapist to produce local muscle contractions without pain or discomfort, and thereafter will the patient monitor the stimulation intensity. Six additional points are selected to strengthen the effect: LI4, ST36, and SP6, and they will be stimulated manually by rotating the needle to evoke needle sensation every 10 min.

The second set of acupuncture points include abdominal points: ST27 bilaterally (EA), CV6 to CV10 (EA); and leg points: SP10 to a non-acupuncture point located 6 cun proximal of patellas medial border (EA) are all connected to an electrical stimulator and stimulated as in the first set of acupuncture points. Six additional points; ST38, liver (LR)3 and pericardium (PC)6, all bilateral, are stimulated manually by rotating the needle to evoke needle sensation every 10 min.

Compliance: If a participant in the acupuncture group deviate considerably form the study protocol, the acupuncturists should inform the study coordinator. Any negative side effects during treatment are recorded.

Metformin: Oral metformin 500 mg three times daily, in total 1500 mg per day [49 50]. To reduce gastrointestinal side-effects of metformin, the dose will be slowly escalated starting with 500 mg daily during the first week, increasing to 500 mg twice per day during the second the week, and 500 mg three times daily, morning, lunch and dinner from the third week in total 16 weeks including the 3 weeks step-up phase (i.e. 4 months). Patients with negative effects can remain 500 mg during the remaining weeks.

Compliance: Empty bottles are handed over to the study coordinator after 16 weeks of treatment and number of tablets are counted. Also, once per month, the study coordinator call the participant and ask her to count the number of tablets there are left in the bottle.

Study Procedure

Screening: The study coordinator describes the study design in detail and written informed consent is collected. Of note, if a participant hesitates to go through tissue sampling as described below, this is not an exclusion criteria. All other outcome measures will be collected and are listed in Table 2.

In all participants, a comprehensive anamnestic interview will be conducted, including menstrual frequency, hirsutism - FG and acne determined by an affirmative answer to the question “Do you have excessive acne? yes or no”, heredity, medication or other diseases. The physical examination including gynaecological examination is performed by transvaginal

ultrasound (PCO morphology: yes or no). Body weight (kg) and body height (cm) are measured in an upright position with light clothing and no shoes. BMI is calculated as body weight (kg) divided by squared body height (m^2). Waist circumference is measured in centimetres at the midpoint between the iliac crest and lower rib margin at the end of expiration, while standing without clothing. Hip circumference is measured in centimetres at the widest point between waist and thighs. Waist-Hip-Ratio (WHR) is calculated as the ratio of waist and hip circumferences. Systolic (SBP) and diastolic blood pressure (DBP) is measured with a semiautomatic blood pressure monitor, and heart rate.

Each woman (PCOS and controls) is given seven questionnaires to be filled in and returned at next (baseline) visit. They are asked to start to register their bleeding periods from now until the end of study.

An appointment for body composition (lean and fat mass and bone mineral density) measure with dual energy x-ray absorptiometry (DXA) is given.

To enable measurements day 6-8 in the menstrual cycle, all women are given information on how to induce withdrawal bleeding with medroxyprogesterone acetate, 10 mg per day for 7 days (participants in Sweden) or dydrogesteron, 20 mg per day for 10 days (participants in China).

Baseline: The baseline visit takes place in the morning after an overnight fast on day 6 – 8 after induced withdrawal bleeding in all women (see above). The time point is selected as the endometrial lining has to be thicker. The questionnaires are returned and checked. Missing information in the questionnaires is checked.

First, a gynaecological examination is performed by transvaginal ultrasound, measuring ovarian size in three dimensions, total antral follicle count (2-9 mm) and endometrial thickness (mm).

Second, if biopsies are taken, local anaesthesia will be placed and an endometrial biopsy is collected and snap frozen. Immediately after, local anaesthesia is placed close to the umbilicus and in the vastus lateralis muscle, fat and muscle biopsy are taken.

Third, a venflon will be placed and fasting blood samples will be drawn for serum and plasma analyses e.g. *genetic* (e.g. next generation sequencing, SNP, methylation), *metabolic* (e.g. lipids, adipokines, inflammatory markers) and *endocrine* (e.g. sex steroids, gonadotropins, growth factors) measures.

Fourth, an oral glucose tolerance test (OGTT) with 75 g glucose will be performed. Blood samples is collected to measure plasma glucose and serum insulin at 0, 30, 60, and 120 min during the OGTT.

At the baseline visit, after OGTT, all participants will receive lifestyle advice by the study coordinator. Patient will be told to register daily number of steps and will receive a step-counter and asked to register menstrual bleeding. If allocated to the electroacupuncture group, time will be booked and treatment started within one week. If randomized to the metformin group, the study drug will be administered and the treatment started the next day. The lifestyle management only group are given appointments for repeated measurements after 4 months and follow-up 4 months later.

Women with PCOS who are randomized are informed that they should use contraception that are non-hormonal.

Follow-up 4 months after last treatment: All baseline measures are repeated after 4 months of treatment and at follow-up 4 months after last treatment.

Statistical analysis

Sample size and power calculations

Sample size calculations are based on t-test between two groups. This is due to the fact that it is the pairwise comparisons that are of main interest (not overall F-test/ANOVA). The result show that 41 women per group, in total 123 women, is enough to prove a difference in Hba1c compared acupuncture + lifestyle management and metformin + lifestyle management respectively, to lifestyle management alone (repeated pairwise t-test) on -1.7 unites (effect size 1.7/2.7SD=0.63) with 80 % power (significance, $p = 0.05$, unadjusted pairwise comparisons).[18 51]

Further, for the mechanistic studies, we estimate that successful tissue samples will be recruited from a minimum 20 participants in each group in Sweden and China respectively, giving a strong power to detect differences.

Minimizing sources of bias

Blinding is not possible given the nature of the intervention. We do not feel it is necessary or ethical to perform sham acupuncture and are confident that the primary outcome is unlikely to be affected by observer bias.

Type of analyses

The statistical analyses will be performed by qualified statisticians and biostatisticians. The data in the RCT will be analysed according to the intent-to-treat principle to investigate differences between the groups.

Clinical outcome measures: Continuous variables will be presented as means ± standard deviations and categorical variables as medians with interquartile ranges. Between group comparisons will be carried out with changes from baseline to after treatment and from baseline to follow-up by ANOVA followed by Dunnet post-hoc test for continuous and Kruskal-Wallis followed by Mann Whitney U-test or by χ^2 tests for categorical variables. In the cross sectional case-control part of the study the Student t-test will be used for continuous variables and Mann Whitney U-test or χ^2 tests for categorical variables and logistic regression when needed.

All statistical analyses of the data will be performed using the SPSS program version 23.0 or higher (SPSS Inc., Chicago, IL, USA), and a *P*-value < 0.0167 will be considered statistically significant in the RCT and *P*-value < 0.05 in the cross sectional part.. All tests are two-sided and adjustments for multiple comparisons will be performed.

Expression and methylation data: These analyses will be adjusted to the technique used. In brief, raw data will be checked and processed and a quality control report will be completed. Different analysis pipelines for traceability and track-ability will be performed. Then extended data analyses, including functional analysis, GeneOntologies, Biological Pathways, Principle Component Analysis (PCA)-analysis, Clustering, Visualizations and mapping against a reference genome, will be performed, and data will be submitted to repositories (i.e. the Array Express: www.ebi.ac.uk/arrayexpress).

Group comparison will be carried out with changes from baseline to after treatment and from baseline to follow-up by Kruskal-Wallis followed by Mann Whitney U-test for expression analyses. In the case-control part of the study, Mann Whitney U-test will be used for expression analyses. False discovery rate (FDR) will be used to correct for multiple testing in the analyses of gene and methylation arrays.

Ethics and Dissemination

The study is performed according to good clinical practice and conducted in accordance with the Declaration of Helsinki. The study has been approved by the Regional Ethical Review

Board of Stockholm, Sweden Dnr: 2015/1656-31/2 and by the Regional Ethical Review Board of Peking University Third Hospital, China Dnr: 2016-212-02. In addition, the Medical Products Agency have approved the study: EudraCT: 2015-004250-18 and the trial is registered at Clinicaltrials.gov: NCT02647827. Reporting of the study results will follow the 2010 revised CONSORT statement and STRICTA.[52 53] Primary outcome data the RCT will be published in a relevant journal together with supporting secondary outcome measurements. Further, secondary outcome measurements will be published in separate papers as well as cross sectional case-control data.

The relevance of this study is that it has potential to uncover new knowledge in the pathophysiology of the disorder and result an additional treatment strategy for insulin resistant in women with PCOS and related diseases, including obesity, insulin resistance, and T2D. Thus, it may have an impact on both genders and does not apply only to women with PCOS.

Trial status

The study was conceived and designed during 2015. The first participant was recruited and randomized in February 2016 in Sweden and September 2016 in China. Number of participants randomized in Sweden: 26 and in China 48 in August 2018. We anticipate that all participants are recruited by the end of 2019 with follow-up done during 2020.

Funding Statement

The work is supported by the Swedish Medical Research Council (Project No. 2014-2775); Adlerbert Research Foundation; Novo Nordisk Foundation (NNF17OC0026724); Strategic Research Programme (SRP) in Diabetes at Karolinska Institutet; Swedish federal government under the Regional agreement on medical training and clinical research (ALF) between Stockholm County Council and Karolinska Institutet (all ESV). In China, this study is supported by the National Key Research and Development Program (2016YFC100021), National Key Technology R&D Program (2015BAI13B06, 2014BAI05B04), National Natural Science Foundation of China (81603446), Beijing Municipal Natural Science Foundation (7174363).The funders have had no role in study design, and will not have any role in data collection and analysis, decision to publish, or preparation of the manuscripts.

Competing interest

The authors declare that they have no competing interests.

Author contribution

ESV conceived and designed the study, drafted the manuscript for important intellectual content and sought funding and ethical approval in Sweden and registered the trial in EudraCT and Clinicaltrials.gov. JQ sought funding and ethical approval in China. HZ, RL, CF, ALH and JQ was involved in the planning and design of the study and critically revised the manuscript and protocols. HZ, DL, WW, HW, CC, SL, ZJH and XJ are involved in the screening, randomization and treatment of participants. All authors read and approved the final manuscript.

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Table 1: Acupuncture points, stimulation, localization, tissue in which needles are inserted, and innervation areas. The two sets will be alternated for every other treatment.

Acupuncture point	Stimulation	Localization	Muscle	Muscle innervation
Set 1				
CV4, *Guan Yuan*	EA	3 cun caudal to the umbilicus	Fibrous tissue, linea alba	L1
CV12, *Zhongwan*	EA	On the midline, 4 cun superior to the umbilicus	Fibrous tissue, linea alba	Th7–8
ST29 Bilateral, *Guilai*	EA	1 cun cranial to the pubic bone and 2 cun lateral of the midline	M. rectus abdominis	Th6–12
ST34 Bilateral, *Futu*	EA	2 cun above the superior lateral border of the patella on the line connecting the anterior superior iliac spine found	M. quadriceps femoris	femoral nerve
ST32 Bilateral, *Liangqiu*	EA	6 cun above the superior lateral border of the patella on the line connecting the anterior superior iliac spine found	M. quadriceps femoris	femoral nerve
SP6 Bilateral, *Sanyinjiao*	DeQi, four times	3 cun proximal to the medial malleolus	Mm. flexor digitorum longus, tibialis posterior	L4–5, S1–2
ST36 Bilateral, *Zusanli*	DeQi, four times	On the anterior lateral side of the leg, 3 cun below *Dubi* (ST35), one finger width (middle finger) from the anterior crest of the tibia	Musculi tibialis anterior	L4–5, S1
LI4 Bilateral, *Hegu*	DeQi, four times	On the highest point at m. interosseus dorsalis	Mm. interosseus dorsalis I, lumbricalis II, adductor pollicis	C8, Th1

Set 2				
CV6, *Qihai*	EA	1.5 cun caudal to the umbilicus	Fibrous tissue, linea alba	Th11
CV10, *Xiawan*	EA	2 cun cranial to the umbilicus	Fibrous tissue, linea alba	Th8
ST27 Bilateral, *Daju*	EA	3 cun cranial to the pubic bone and 2 cun lateral to the midline	M. rectus abdominis	Th6–12
Extra meridian point Bilateral	EA	6 cun above the patella in line with SP10	M. quadriceps femoris	L2–L4
SP10 Bilateral, *Xuehai*	EA	With the knee flexed, on the medial side of the thigh 2 cun above the superior medial corner of the patella on the prominence of the medial head of the quadriceps muscle of the thigh	M. quadriceps femoris	L2–L4
ST38 Bilateral, *Sanyinjiao*	DeQi four times	Between lateral malleolus and knee joint, 1 finger from tibiae crist	Musculi tibialis anterior	L4–5, S1
LR3 Bilateral, *Taichong*	DeQi four times	Between metatarsal I & II, just distal to the caput	M. Interosseus dorsalis I	S2–3
PC6 Bilateral, *Neiguan*	DeQi four times	2 cun proximal to the processus styloideus radii, between the tendons of the palmaris longus and the flexor carpi radialis	M. flexor digitorum superficialis	C8, Th1

C: Cervical vertebra; CV: Conception vessel; L: Lumbar vertebra; LI: Large intestine; LR: Liver; PC: Pericardium; S: Sacral vertebra; SP: Spleen; ST: Stomach; Th: Thoracic vertebra.

Table 2. Overview of the study visits.

	Screening visit	**Base line visit**	**Month**				**Follow-up after 4 months of treatment**	**Follow-up 4 months after last treatment**
			1st	2nd	3rd	4th		
Anthropometry: Body composition (weight, height, waist circumference, hip circumference), FG/acne, blood pressure	X						X	X
Menstrual cycle diary	X	X	X	X	X	X	X	X
Questionnaires: EQ-5D, SF36, PCOSQ, CPRS-SA*, Zung SAS#, Zung SDS#, IPAQ, TFEQ-R21, QEWP-R*	X						X	X
Transvaginal ultrasound		X					X	X
Metabolic measures: Fasting blood samples for glucose, insulin, HbA1c, c-peptide, OGTT. Adipokines, lipid profile (LDL, HDL, NEFA) and inflammatory markers		X					X	X
DXA		X					X	X
Endocrine measures: Fasting blood samples for sex steroids, SHBG, LH, FSH, AMH, prolactin, TSH, T4		X					X	X
Tissue and whole blood collection		X					X	X

Antimüllerian hormone (AMH); dual energy x-ray absorptiometry (DXA); EuroQol-5 dimension (EQ-5D); Ferriman–Gallwey score (FG); follicle stimulating hormone (FSH); high density lipoprotein (HDL); hsCRP, International Physical Activity Questionnaire (IPAQ); low density lipoprotein (LDL), luteinizing hormone (LH); non-esterified fatty acids (NEFA); norepinephinre (NE), oral glucose tolerance test (OGTT); polycystic ovary syndrome questionnaire (PCOSQ); Questionnaire of Eating and Weight Patterns-Revised (QEWP-R); sex hormone binding globulin

(SHBG); short form-36 (SF36); Three-Factor Eating Questionnaire (TFEQ-R21); thyroid stimulating hormone (TSH); thyroxine (T4); Zung Self-Rating Anxiety Scale (Zung SAS); Zung Self-Rating Depression Scale (Zung SDS).

* only in Sweden and # only in China.

BMJ Open

Acupuncture or metformin to improve insulin resistance in women with polycystic ovary syndrome: Study protocol of a combined multinational cross sectional case-control study and a randomized controlled trial

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2018-024733.R2
Article Type:	Protocol
Date Submitted by the Author:	07-Nov-2018
Complete List of Authors:	Stener-Victorin, Elisabet; Karolinska Institutet, Department of Physiology and Pharmacology Zhang, Haolin; Peking University Third Hospital, Department of Traditional Chinese Medicine (TCM) Rong, LI; Peking University Third Hospital, OB & GYN Friden, Cecilia; Karolinska Institutet, 5. Department of Neurobiology, Care Sciences and Society, Division of Physiotherapy Li, Dong; Peking University Third Hospital, Department of Traditional Chinese Medicine (TCM) Wang, Wei; Peking University Third Hospital, 4. Department of Obstetrics and Gynecology, Peking University Third Hospital, Beijing, China Wang, Haining ; Peking University Third Hospital, Department of Endocrinology and Metabolism, Peking University Third Hospital, Beijing, China Chang , Cuiqing; Peking University Third Hospital, Institute of Sports Medicine Li , Shi; Peking University Third Hospital, 3. Center for Reproductive Medicine, Department of Obstetrics and Gynecolog Huo , ZeJun ; Peking University Third Hospital, Research Center of Clinical Epidemiology Zhang, Hua; Peking University Third Hospital, Research Center of Clinical Epidemiology Ji, Xiaolan ; Peking University Third Hospital, Department of Traditional Chinese Medicine (TCM) Linden-Hirschberg, Angelica; Karolinska Institutet, Department of Obstetrics and Gynecology Jie, Qiao; Peking University Third Hospital, Center for Reproductive Medicine, Department of Obstetrics and Gynecology andDepartment of Obstetrics and Gynecology, Peking University Third Hospital, Beijing, China
Primary Subject Heading:	Diabetes and endocrinology
Secondary Subject Heading:	Complementary medicine
Keywords:	glucose homeostasis, insulin resistance, acupuncture, life style, polycystic ovary syndrome

Acupuncture or metformin to improve insulin resistance in women with polycystic ovary syndrome: Study protocol of a combined multinational cross sectional case-control study and a randomized controlled trial

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Short title: Acupuncture or metformin for insulin resistance in PCOS

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Abstract

Introduction: Polycystic ovary syndrome (PCOS) is linked to hyperinsulinemia and insulin resistance with dysfunctional glucose metabolism. Pilot studies suggests that acupuncture treatment with combined manual and low-frequency electrical stimulation (electroacupuncture, EA) of the needles decrease circulating glycated hemoglobulin (HbA1c) and homeostatic model assessment-insulin resistance (HOMA-IR). Therefore, we here aim to investigate if acupuncture treatment or metformin together with life style or life style management alone improve insulin sensitivity and related symptoms in overweight/obese women with PCOS.

Methods and analysis: This is a two centre multinational (Sweden and China), cross-sectional case-control study combined with an open-labelled randomized controlled trial (RCT). Participants are randomized to one of three groups: 1) EA 2-3 times/week during 4 months + lifestyle management; 2) metformin, 500 mg, three/day during 4 months + lifestyle management; or 3) life style management alone. The primary outcome measure in the RCT is changes in Hba1C. A total of 123 obese overweight women with PCOS will be enrolled and randomized into one of the three groups with a target power of at least 80% and 5% significance level based on two-sided tests.

Ethics and dissemination: The study has been approved by the Regional Ethical Review Board of Stockholm and of Peking University Third Hospital, China. Primary outcome data of the RCT will be published in a relevant journal together with supporting secondary outcome measurements. Further, outcome measurements will be published in separate papers as well as case-control data.

Expected results: We anticipate that EA and metformin, both with lifestyle management are equally effective and superior to lifestyle management alone for improvement of glycemic control.

Trial registration: Clinicaltrials.gov: NCT02647827 and EudraCT: 2015-004250-18.

Key words: glucose homeostasis; insulin resistance; metformin; electroacupuncture; lifestyle

Strength and Limitations

1. A strength of this trial is that all patients will benefit from receiving treatment, all of which alone and/or in combination may offer an increased chance for improved metabolic function and reproductive health.

2. It has the potential to gain deeper insight into the pathophysiology of polycystic ovary syndrome (PCOS), and to uncover new knowledge for treatment of insulin resistant in related diseases, including obesity, insulin resistance, and type 2 diabetes (T2D).

3. The results from the present study have the potential to immediately be implemented into the healthcare system since it has previously been shown to be cost-effective and to have few negative side effects.

4. A potential limitation is that metformin might cause side-effects such as diarrhoea, nausea/vomiting, flatulence, asthenia, indigestion, abdominal discomfort and headache and acupuncture local skin irritation, discomfort, and vasovagal reactions during the procedure.

Background

Polycystic Ovary Syndrome (PCOS) is the most common endocrine and metabolic disorder in women of reproductive age and is characterized by anovulation, hyperandrogenism and metabolic dysfunction.[1] Women with PCOS have a 3 to 7-fold increased risk of developing type 2 diabetes (T2D), and with younger onset, PCOS increases cardiovascular risk factors[1-6]. Independent of body weight, insulin sensitivity is ~40% lower in women with PCOS than in healthy women, and impaired glucose regulation, insulin resistance and reduced insulin responsiveness have been attributed to defects in insulin signalling in adipocytes and skeletal muscle.[7 8] Of note, obesity aggravates all symptoms related to PCOS.

Despite detrimental impact on women's health, the aetiology of PCOS is not well understood. Genetic, epigenetic, and environmental factors have all been implicated in its development. Emerging evidence suggests that PCOS originates, at least in part, in fetal life,[9 10] and elevated maternal androgens have been implicated to play a role, however the mechanisms are largely unknown.[11 12] Of interest is that we have found that women with PCOS have multiple transcriptional and epigenetic changes in adipose tissue that are relevant for development of the disease.[13] Further, twin studies suggest that genetic influences explain >70% of PCOS pathogenesis.[14] However, whether genetic or epigenetic alterations in target tissues e.g. adipose tissue, skeletal muscle and endometrium contribute to development of metabolic disease requires further investigation.

Women with PCOS require long-term individualized treatment programs. Pharmacological treatments, including the glucose reducing drug metformin, have limitations related to adverse effects and patient compliance. Therefore, there is a need for inexpensive and easily administered treatments with few negative side-effects. Lifestyle management is the first line treatment eventually with addition of metformin for improving whole body glucose homeostasis and preventing type 2 diabetes (T2D).[1 15-17] Interestingly, five weeks of acupuncture with combined manual and low-frequency electrical stimulation has in a pilot study been shown to improve whole body glucose homeostasis in insulin resistant women with PCOS.[18] The pilot study was an uncontrolled trial and it is therefore of importance to compare the effect of acupuncture with first line treatment to investigate the effectiveness.

Whilst pharmacological treatment strategies have shown efficacy, importantly, there is a need for Comparative Effectiveness Research (CER) to strengthen the evidence base for clinical and policy decision-making [19]. Therefore we aim to compare the effect of pharmacological first-line treatment, metformin, with a non-pharmacological treatment strategy, acupuncture

(both together with lifestyle management), with lifestyle management for improvement and prevention of metabolic dysfunction and related symptoms in insulin resistant women with PCOS.

Our main hypothesis is that acupuncture and metformin (both treatments combined with lifestyle management) are superior to lifestyle management alone in improving whole body glucose regulation in insulin resistant women with PCOS. Secondary hypotheses are that these treatments have the potential to improve metabolic- and endocrine measures, quality of life and symptom of anxiety and depression, and to restore epigenetic and molecular alterations in target tissues (endometrial-, adipose-, and skeletal muscle tissue) and thus have the potential to improve and potentially prevent the development of metabolic alterations including T2D.

Thus, the purpose of this study is twofold, first we aim to gain deeper insight into the pathophysiology of PCOS in a cross sectional case-control study by comparing women with PCOS with women without PCOS matched for age, weight and BMI, and secondly we aim to perform a prospective RCT of women with PCOS.

Study design

This is a two centre multinational prospective trial with a cross-sectional case-control part and an open-labelled RCT with a comparative effectiveness design. The interventions to be tested are 1) Electroacupuncture during 4 months + lifestyle management; 2) Metformin during 4 months + lifestyle management; or 3) Lifestyle management alone which will be available for participants in all three groups. Participants will be enrolled at Karolinska Institutet and Karolinska University Hospital, Stockholm, Sweden and at Peking University Hospital, Beijing China respectively.

Randomisation and treatment allocation

The randomization is stratified across the factors age and BMI and is also separated by study site with a balanced allocation ratios 1:1:1. Randomization is performed in blocks with a variable block size between 3 and 15; e.g. First there is a block of 12, when it is full it is followed by a block of 9 and thereafter a block of 3. The order of the block sizes are unknown to the participating study sites and also differs among the strata's.
Each study site (Stockholm and Beijing) use the same randomization and electronic case report form (eCRF). A web-based randomization program (https://data.dynareg.se/pia2/Default.aspx) has been generated to ensure allocation

concealment. The study coordinators log on the web-based system to randomize eligible patients. All women who enter the study will be logged and given a unique study number. Blinding or masking of the intervention will not be possible because of the nature of the intervention. Importantly, however, the assessor will be blinded to the patients' group assignment.

Patient and Public Involvement

Patients and or public was not involved in the design of this study.

Study Objectives

Primary Objective

1. To determine the clinical effectiveness of 4 months of 1) electroacupuncture + lifestyle management and 2) metformin + lifestyle management, compared to 3) lifestyle management only for improvement of glucose regulation assessed by Hba1c levels.

Secondary Objectives

1. To evaluate changes in secondary metabolic measures including the insulin response to glucose assessed by calculating the area under the curve ($AUC_{insulin}$) during the oral glucose tolerance test (OGTT), fasting insulin, glucose, calculation of homeostatic model assessment (HOMA)-IR and-HOMA-B (*i.e.* the Islet β-cell function) and the assessment of e.g. adipokines, lipid profile, body size and proportions and body fat distribution.
2. To determine changes in gene expression and DNA methylation profiles related to insulin sensitivity in fat, muscle and endometrial tissue biopsies, and biomarkers in whole blood.
3. To evaluate endocrine measures including menstrual pattern and ovulation frequency, circulating hormones (e.g. sex steroids, AMH, gonadotropins).
4. To determine changes in women's health related quality of life (HRQoL), symptoms of anxiety and depression, dieting and eating patterns, and negative side-effects.

Outcome Measurements

Outcome measures will be collected at:

1. Baseline
2. After 4 months of intervention

3. Follow-up 4 months after last treatment

Primary Outcome

Changes from baseline to after 4 months of treatment in HbA1c comparing 1) acupuncture + lifestyle management and 2) metformin + lifestyle management, respectively with 3) lifestyle management only. In the cross sectional study, difference in HbA1c between cases and controls.

Secondary Outcome

Changes from baseline to after 4 months of treatment and from baseline to the 4-month follow-up comparing 1) acupuncture + lifestyle management, and 2) metformin + lifestyle management, respectively, with 3) lifestyle management only, and in the cross sectional study, difference between cases and controls in the following variables:

- *Body composition*: In addition to weight, height and waist circumference, women will be examined by DXA to measure lean and fat mass and bone mineral density using a Lunar Prodigy Advance whole body scanner (GE Medical Systems) [20].
- *Metabolic measures:* Insulin response to glucose during the OGTT (AUC using the trapezoidal rule), and direct analyses of fasting blood samples of insulin an glucose to enable calculation of HOMA-IR [fasting insulin (μU/mL) × fasting glucose (mmol/L)] / 22.5)], and HOMA-B /islet β-cell function [20 × fasting insulin (mU/mL) / (fasting plasma glucose (mmol/L) − 3.5)] [21]. Further, fasting blood samples are collected and saved for later analyses of e.g. C-peptide and calculation of C-peptide index [Fasting C-peptide (nmol/L)/ f-glucose (mmol/L) x 100] [22], for analyses of adipokines, inflammatory markers, non-esterified fatty acids (NEFA), total cholesterol, triglycerides, high density lipoprotein (HDL), low density lipoprotein (LDL), high sensitive CRP, catecholamine's and metabolites analysed on a split-fraction HPLC-ED system [23].
- *Endocrine measures*: Menstrual frequency: Participants will be asked to note date of menstruation which will be reported to the study coordinator once per week by text message and every 4th week by phone. Ovarian morphology antral follicle count and ovarian volume. Blood samples will be drawn for analyses of sex steroids by the validated gas- and liquid chromatography/tandem mass spectroscopy technique, as well as sex hormone binding globulin (SHBG), luteinizing hormone (LH), follicle stimulating hormone (FSH), antimüllerian hormone (AMH), prolactin, thyroid stimulating hormone (TSH) and free thyroxine (T4).

- *Tissue and whole blood collection:* Whole blood will be collected for DNA and microRNA analyses. Endometrial, fat and skeletal muscle tissue biopsies will be collected at baseline (cases and controls), after 4 months of treatment and at follow-up 4 months after treatment in women with PCOS, snap frozen in liquid nitrogen within 30 s and stored at -80°C for later analyzes. Fat cells will be isolated for determination of adipocyte size and distribution. Part of tissue biopsies will also be isolated for *in vitro* experiments.[24] Deep RNA, microRNA and/or bisulfite sequencing will be performed with the latest available technology.
- *Health related quality of life:* Will be determined by quality of life by EuroQol-5 dimension (EQ-5D),[25 26] short form-36 (SF36),[27 28] and polycystic ovary syndrome questionnaire (PCOSQ).[29 30]
- *Symptoms of anxiety and depression* will be assessed by the self-reported version of the Comprehensive Psychopathological Rating Scale for Affective Syndromes (CPRS-S-A)[31] to assess psychiatric symptoms within a time frame of the last 3 days in Sweden. For the purpose of this study, two scales will be extracted from the CPRS-S-A,[31] the Brief Scale for Anxiety (BSA-S)[32] and the Montgomery Åsberg Depression Rating Scale (MADRS-S).[33-35] In China will the Zung symptom depressions score (SDS) and Zung symptom anxiety score (SAS) be used [36 37]. Depression symptoms of potential clinical relevance is for MADR-S ≥11 and for Zung SDS ≥ 0.5 (Depressive index), Anxiety symptoms of potential clinical relevance is for BSA-S ≥11 and for Zung SAS ≥50 (Standard total score),
- *Physical Activity:* International Physical Activity Questionnaire (IPAQ) will be used to assess degree of physical activity.[38 39] In addition, one text message per week will be sent to the participants by the study coordinators asking of number of steps the last week when asking for menstrual bleeding (date).
- *Eating questionnaire and eating pattern:* Only assessed at baseline using the self-reported version of the Three-Factor Eating Questionnaire (TFEQ-R21),[40 41] and Questionnaire of Eating and Weight Patterns-Revised (QEWP-R) to measure eating behaviour (Sweden only).[42]
- *Side-effects and adverse events* will be continuously and equally recorded in each study arm. One text message per week will be sent to all participants by the study coordinators in which they are asked to report (in addition to number of steps) any side effects or

adverse events. All participants will receive a phone call every 4th week by the study coordinator and will be asked about side effects or adverse events.

Participants

Eligible women will be identified by their clinician, or by local newspaper advertisements, and invited to participate in the trial. Each participant will be given written and oral information and asked for her signed informed consent to be randomized and followed-up by research staff. The cross sectional case-control part of the study equals baseline measurements for women with PCOS and controls. If a patient do not adhere to the frequent treatment this will be clearly tracked as the treatment may still be effective.

Inclusion criteria – women with PCOS

1. Age ≥18 to ≤40 years
2. Body mass index (BMI) ≥25 to ≤40 given that 95% of all women with PCOS with a BMI ≥25 are insulin resistant.[43 44]
3. PCOS diagnosis according to Rotterdam criteria 2003 [45], with at least two of the following three symptoms: Clinical and/or biochemical signs of hyperandrogenism (hirsutism or acne); oligo/amenorrhea; and/or polycystic ovaries (PCOS). Biochemical hyperandrogenism is defined by total testosterone >1.2 nmol/L or a free androgen index (FAI)>5.[46] Hirsutism is defined as a self-reported Ferriman-Gallwey (FG) score ≥8 (≥5 Asian).[47 48] Acne is defined by a positive response to the question *Do you have acne?* Oligomenorrhea is defined as an intermenstrual interval >35 days and <8 menstrual bleedings in the past year. Amenorrhea as <3 cycles per year. PCO is defined by transvaginal ultrasound with ≥12 follicles 2–9 mm and/or ovarian volume ≥10 ml in one or both ovaries.
4. Willing to sign the consent form.

Inclusion criteria – controls

Controls should have BMI ≥25 to ≤40, regular cycles with 28 days ± 2 days, and no signs of hyperandrogenism. They are excluded if they have menstrual irregularities, signs of hyperandrogenism (FG >4), or evidence of PCO morphology on ultrasound.

Exclusion criteria for all women

1. Exclusion of other endocrine disorders such as non-classic congenital adrenal hyperplasia (17-hydroxyprogesterone < 3nmol/L), androgen secreting tumors or suspected Cushing's syndrome.
2. Having known renal disease (creatinine clearance < 60 mL/min), hepatic insufficiency, autoimmune disorders or cancer.
3. Any acute condition with potential to alter renal function or cause tissue hypoxia.
4. Type I diabetes.
5. Pharmacological treatment (cortizon, antidepressant, other antidiabetic treatment such as insulin and acarbose, hormonal contraceptives, hormonal ovulation induction or other drugs judged by discretion of investigator) within 12 weeks. Depo Provera or similar within 6 months.
6. Hypersensitivity to metformin hydrochloride or to any of the excipients.
7. Blood pressure >160 / 100 mmHg
8. Pregnancy or breastfeeding the last 6 months
9. Acupuncture the last 2 months
10. Daily smoking and alcoholic intake
11. Language barrier or disabled person with reduced ability to understand the information given.

In total 50 controls will be matched at baseline (age, weight and BMI) to women with PCOS. Controls will undergo screening and baseline visit, but will not be randomized to any treatment.

Interventions

Participants fulfilling the inclusion criteria will be randomized to one of three groups after baseline measurements:

1. Electroacupuncture 2-3 times/week during 4 months + lifestyle management
2. Metformin, 500 mg, three times/day during 4 months + lifestyle management
3. Lifestyle management alone which will be available for participants in all three groups.

Lifestyle management: All women will receive lifestyle management instructions at the baseline visit, before randomization. The lifestyle management involves one initial

counselling session in connection with the baseline visit, which includes information about the importance of weight management, healthy diet and physical activity. Focus will be on the importance of physical activity. Each participant will receive a book with lifestyle advice about weight reduction, maintenance and physical activity following WHO recommendations. All participants will receive a text message once weekly to respond number of step collected by their smart phone or step counter during the last week and if they have had any menstrual bleedings. Once every fourth week, study coordinator will call to the participant and ask about number of step last week, menstruation and compliance and side-effects.

Electroacupuncture: Women randomized to receive acupuncture will start their treatment within one week after baseline measurements. The rationale of the acupuncture protocol is based on Western Medical Acupuncture theories. We will use a fixed acupuncture protocol following the two pilot studies: ClinicalTrial.gov NCT01457209 and NCT02026323 with two modifications. First, the treatment period will be 16 weeks (i.e. 4 months) compared to 5 weeks and 6 months in the previous pilot studies. Second, the treatment frequency will be 2 to 3 times per week during 16 weeks, i.e. in total 32 to 48 acupuncture treatments over 16 weeks. The rational for these changes is that the procedure is time-consuming for the patients and this will increase the feasibility and likely reduce the number of dropouts. Acupuncture treatment will be given by registered physiotherapists or medical doctors educated in theoretical and practical acupuncture and trained to follow the fixed protocol. Disposable, single-use, sterilized CE marked needles made of stainless steel, 0.25 x 30 mm and 0.30 x 40/50 mm (XENO, HEGU Svenska AB, Landsbro, Sweden and in China Huatuo, Suzhou Medical Co Ltd, China) will be inserted to a depth of 15–40 mm in segmental acupuncture points located in abdominal and leg muscles, with innervations corresponding to the ovaries and the pancreas. Two sets of acupuncture points will be alternated every second treatment (Table 1). The first set of acupuncture points include points located in abdominal muscles: conception vessel (CV)4, CV12, stomach (ST)29 bilaterally, and in quadriceps muscle, ST32 and ST34, and in the muscles below the knee, spleen (SP)6, and ST36 bilaterally. In addition, needles are placed in the hand, large intestinal (LI)4, bilateral. All needles will be stimulated manually when inserted. CV4 and 12, ST29 bilateral, ST32 and ST34 bilateral will be connected to an electrical stimulator and stimulated with low-frequency EA of 2 Hz (Stimulators used in Sweden: Export Abteilung, Schwa-Medico GmbH, Wetzlarer Str. 41-43;35630 Ehringshausen and in China: Shanghai Huayi Electric

Acupuncture Instrument: G6805-1A) for 30 min at each treatment. The intensity will be adjusted by the physiotherapist to produce local muscle contractions without pain or discomfort, and thereafter will the patient monitor the stimulation intensity. Six additional points are selected to strengthen the effect: LI4, ST36, and SP6, and they will be stimulated manually by rotating the needle to evoke needle sensation every 10 min.

The second set of acupuncture points include abdominal points: ST27 bilaterally (EA), CV6 to CV10 (EA); and leg points: SP10 to a non-acupuncture point located 6 cun proximal of patellas medial border (EA) are all connected to an electrical stimulator and stimulated as in the first set of acupuncture points. Six additional points; ST38, liver (LR)3 and pericardium (PC)6, all bilateral, are stimulated manually by rotating the needle to evoke needle sensation every 10 min.

Compliance: If a participant in the acupuncture group deviate considerably form the study protocol, the acupuncturists should inform the study coordinator. Any negative side effects during treatment are recorded.

Metformin: Oral metformin 500 mg three times daily, in total 1500 mg per day [49 50]. To reduce gastrointestinal side-effects of metformin, the dose will be slowly escalated starting with 500 mg daily during the first week, increasing to 500 mg twice per day during the second the week, and 500 mg three times daily, morning, lunch and dinner from the third week in total 16 weeks including the 3 weeks step-up phase (i.e. 4 months). Patients with negative effects can remain 500 mg during the remaining weeks.

Compliance: Empty bottles are handed over to the study coordinator after 16 weeks of treatment and number of tablets are counted. Also, once per month, the study coordinator call the participant and ask her to count the number of tablets there are left in the bottle.

Study Procedure

Screening: The study coordinator describes the study design in detail and written informed consent is collected. Of note, if a participant hesitates to go through tissue sampling as described below, this is not an exclusion criteria. All other outcome measures will be collected and are listed in Table 2.

In all participants, a comprehensive anamnestic interview will be conducted, including menstrual frequency, hirsutism - FG and acne determined by an affirmative answer to the question “Do you have excessive acne? yes or no”, heredity, medication or other diseases. The physical examination including gynaecological examination is performed by transvaginal

ultrasound (PCO morphology: yes or no). Body weight (kg) and body height (cm) are measured in an upright position with light clothing and no shoes. BMI is calculated as body weight (kg) divided by squared body height (m^2). Waist circumference is measured in centimetres at the midpoint between the iliac crest and lower rib margin at the end of expiration, while standing without clothing. Hip circumference is measured in centimetres at the widest point between waist and thighs. Waist-Hip-Ratio (WHR) is calculated as the ratio of waist and hip circumferences. Systolic (SBP) and diastolic blood pressure (DBP) is measured with a semiautomatic blood pressure monitor, and heart rate.

Each woman (PCOS and controls) is given seven questionnaires to be filled in and returned at next (baseline) visit. They are asked to start to register their bleeding periods from now until the end of study.

An appointment for body composition (lean and fat mass and bone mineral density) measure with dual energy x-ray absorptiometry (DXA) is given.

To enable measurements day 6-8 in the menstrual cycle, all women are given information on how to induce withdrawal bleeding with medroxyprogesterone acetate, 10 mg per day for 7 days (participants in Sweden) or dydrogesteron, 20 mg per day for 10 days (participants in China).

Baseline: The baseline visit takes place in the morning after an overnight fast on day 6 – 8 after induced withdrawal bleeding in all women (see above). The time point is selected as the endometrial lining has to be thicker. The questionnaires are returned and checked. Missing information in the questionnaires is checked.

First, a gynaecological examination is performed by transvaginal ultrasound, measuring ovarian size in three dimensions, total antral follicle count (2-9 mm) and endometrial thickness (mm).

Second, if biopsies are taken, local anaesthesia will be placed and an endometrial biopsy is collected and snap frozen. Immediately after, local anaesthesia is placed close to the umbilicus and in the vastus lateralis muscle, fat and muscle biopsy are taken.

Third, a venflon will be placed and fasting blood samples will be drawn for serum and plasma analyses e.g. *genetic* (e.g. next generation sequencing, SNP, methylation), *metabolic* (e.g. lipids, adipokines, inflammatory markers) and *endocrine* (e.g. sex steroids, gonadotropins, growth factors) measures.

Fourth, an oral glucose tolerance test (OGTT) with 75 g glucose will be performed. Blood samples is collected to measure plasma glucose and serum insulin at 0, 30, 60, and 120 min during the OGTT.

At the baseline visit, after OGTT, all participants will receive lifestyle advice by the study coordinator. Patient will be told to register daily number of steps and will receive a step-counter and asked to register menstrual bleeding. If allocated to the electroacupuncture group, time will be booked and treatment started within one week. If randomized to the metformin group, the study drug will be administered and the treatment started the next day. The lifestyle management only group are given appointments for repeated measurements after 4 months and follow-up 4 months later.

Women with PCOS who are randomized are informed that they should use contraception that are non-hormonal.

Follow-up 4 months after last treatment: All baseline measures are repeated after 4 months of treatment and at follow-up 4 months after last treatment.

Statistical analysis

Sample size and power calculations

Sample size calculations are based on t-test between two groups. This is due to the fact that it is the pairwise comparisons that are of main interest (not overall F-test/ANOVA). The result show that 41 women per group, in total 123 women, is enough to prove a difference in Hba1c compared acupuncture + lifestyle management and metformin + lifestyle management respectively, to lifestyle management alone (repeated pairwise t-test) on -1.7 unites (effect size 1.7/2.7SD=0.63) with 80 % power (significance, $p = 0.05$, unadjusted pairwise comparisons).[18 51]

Further, for the mechanistic studies, we estimate that successful tissue samples will be recruited from a minimum 20 participants in each group in Sweden and China respectively, giving a strong power to detect differences.

Minimizing sources of bias

Blinding is not possible given the nature of the intervention. We do not feel it is necessary or ethical to perform sham acupuncture and are confident that the primary outcome is unlikely to be affected by observer bias.

Type of analyses

The statistical analyses will be performed by qualified statisticians and biostatisticians. The data in the RCT will be analysed according to the intent-to-treat principle to investigate differences between the groups.

Clinical outcome measures: Continuous variables will be presented as means ± standard deviations and categorical variables as medians with interquartile ranges. Between group comparisons will be carried out with changes from baseline to after treatment and from baseline to follow-up by ANOVA followed by Dunnet post-hoc test for continuous and Kruskal-Wallis followed by Mann Whitney U-test or by χ^2 tests for categorical variables. In the cross sectional case-control part of the study the Student t-test will be used for continuous variables and Mann Whitney U-test or χ^2 tests for categorical variables and logistic regression when needed.

All statistical analyses of the data will be performed using the SPSS program version 23.0 or higher (SPSS Inc., Chicago, IL, USA), and a *P*-value < 0.0167 will be considered statistically significant in the RCT and *P*-value < 0.05 in the cross sectional part.. All tests are two-sided and adjustments for multiple comparisons will be performed.

Expression and methylation data: These analyses will be adjusted to the technique used. In brief, raw data will be checked and processed and a quality control report will be completed. Different analysis pipelines for traceability and track-ability will be performed. Then extended data analyses, including functional analysis, GeneOntologies, Biological Pathways, Principle Component Analysis (PCA)-analysis, Clustering, Visualizations and mapping against a reference genome, will be performed, and data will be submitted to repositories (i.e. the Array Express: www.ebi.ac.uk/arrayexpress).

Group comparison will be carried out with changes from baseline to after treatment and from baseline to follow-up by Kruskal-Wallis followed by Mann Whitney U-test for expression analyses. In the case-control part of the study, Mann Whitney U-test will be used for expression analyses. False discovery rate (FDR) will be used to correct for multiple testing in the analyses of gene and methylation arrays.

Safety analysis

Adverse events will be categorized and the percentage of patients experiencing adverse events and serious adverse events during the treatment period and follow-up period will be

documented and reported to the Data and Safety Monitoring Board (DSMB). These are reviewed every fourth month, and serious adverse events will be immediately handled.

Data management and quality control of data

We use both paper CRF and web-based eCRF to manage individual participant data. Quality control are handled at two levels. First the investigators are required to ensure the accuracy when imputing data into the eCRF. Second, data monitoring and validation will be carried out by an independent person not involved in data collection.

Ethics and Dissemination

The study is performed according to good clinical practice and conducted in accordance with the Declaration of Helsinki. The study has been approved by the Regional Ethical Review Board of Stockholm, Sweden Dnr: 2015/1656-31/2 and by the Regional Ethical Review Board of Peking University Third Hospital, China Dnr: 2016-212-02. In addition, the Medical Products Agency have approved the study: EudraCT: 2015-004250-18 and the trial is registered at Clinicaltrials.gov: NCT02647827. Reporting of the study results will follow the 2010 revised CONSORT statement and STRICTA.[52 53] Primary outcome data the RCT will be published in a relevant journal together with supporting secondary outcome measurements. Further, secondary outcome measurements will be published in separate papers as well as cross sectional case-control data.

The relevance of this study is that it has potential to uncover new knowledge in the pathophysiology of the disorder and result an additional treatment strategy for insulin resistant in women with PCOS and related diseases, including obesity, insulin resistance, and T2D. Thus, it may have an impact on both genders and does not apply only to women with PCOS.

Trial status

The study was conceived and designed during 2015. The first participant was recruited and randomized in February 2016 in Sweden and September 2016 in China. Number of participants randomized in Sweden: 26 and in China 48 in August 2018. We anticipate that all participants are recruited by the end of 2019 with follow-up done during 2020.

Protocol versions

Updated 1: 2015-12-16

Updated 2: 2016-02-11

Updated 3: 2016-12-12

Funding Statement

The work is supported by the Swedish Medical Research Council (Project No. 2018-0243599; Adlerbert Research Foundation; Novo Nordisk Foundation (NNF18OC0033992); Strategic Research Programme (SRP) in Diabetes at Karolinska Institutet; Swedish federal government under the Regional agreement on medical training and clinical research (ALF) between Stockholm County Council and Karolinska Institutet (all ESV). In China, this study is supported by the National Key Research and Development Program (2016YFC100021), National Key Technology R&D Program (2015BAI13B06, 2014BAI05B04), National Natural Science Foundation of China (81603446), Beijing Municipal Natural Science Foundation (7174363).The funders have had no role in study design, and will not have any role in data collection and analysis, decision to publish, or preparation of the manuscripts.

Competing interest

The authors declare that they have no competing interests.

Author contribution

ESV conceived and designed the study, drafted the manuscript for important intellectual content and sought funding and ethical approval in Sweden and registered the trial in EudraCT and Clinicaltrials.gov. JQ sought funding and ethical approval in China. HZ, RL, CF, ALH and JQ was involved in the planning and design of the study and critically revised the manuscript and protocols. HZ, DL, WW, HW, CC, SL, ZJH and XJ are involved in the screening, randomization and treatment of participants. All authors read and approved the final manuscript.

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Table 1: Acupuncture points, stimulation, localization, tissue in which needles are inserted, and innervation areas. The two sets will be alternated for every other treatment.

Acupuncture point	Stimulation	Localization	Muscle	Muscle innervation
Set 1				
CV4, *Guan Yuan*	EA	3 cun caudal to the umbilicus	Fibrous tissue, linea alba	L1
CV12, *Zhongwan*	EA	On the midline, 4 cun superior to the umbilicus	Fibrous tissue, linea alba	Th7–8
ST29 Bilateral, *Guilai*	EA	1 cun cranial to the pubic bone and 2 cun lateral of the midline	M. rectus abdominis	Th6–12
ST34 Bilateral, *Futu*	EA	2 cun above the superior lateral border of the patella on the line connecting the anterior superior iliac spine found	M. quadriceps femoris	femoral nerve
ST32 Bilateral, *Liangqiu*	EA	6 cun above the superior lateral border of the patella on the line connecting the anterior superior iliac spine found	M. quadriceps femoris	femoral nerve
SP6 Bilateral, *Sanyinjiao*	DeQi, four times	3 cun proximal to the medial malleolus	Mm. flexor digitorum longus, tibialis posterior	L4–5, S1–2
ST36 Bilateral, *Zusanli*	DeQi, four times	On the anterior lateral side of the leg, 3 cun below *Dubi* (ST35), one finger width (middle finger) from the anterior crest of the tibia	Musculi tibialis anterior	L4–5, S1
LI4 Bilateral, *Hegu*	DeQi, four times	On the highest point at m. interosseus dorsalis	Mm. interosseus dorsalis I, lumbricalis II, adductor pollicis	C8, Th1

Set 2				
CV6, *Qihai*	EA	1.5 cun caudal to the umbilicus	Fibrous tissue, linea alba	Th11
CV10, *Xiawan*	EA	2 cun cranial to the umbilicus	Fibrous tissue, linea alba	Th8
ST27 Bilateral, *Daju*	EA	3 cun cranial to the pubic bone and 2 cun lateral to the midline	M. rectus abdominis	Th6–12
Extra meridian point Bilateral	EA	6 cun above the patella in line with SP10	M. quadriceps femoris	L2–L4
SP10 Bilateral, *Xuehai*	EA	With the knee flexed, on the medial side of the thigh 2 cun above the superior medial corner of the patella on the prominence of the medial head of the quadriceps muscle of the thigh	M. quadriceps femoris	L2–L4
ST38 Bilateral, *Sanyinjiao*	DeQi four times	Between lateral malleolus and knee joint, 1 finger from tibiae crist	Musculi tibialis anterior	L4–5, S1
LR3 Bilateral, *Taichong*	DeQi four times	Between metatarsal I & II, just distal to the caput	M. Interosseus dorsalis I	S2–3
PC6 Bilateral, *Neiguan*	DeQi four times	2 cun proximal to the processus styloideus radii, between the tendons of the palmaris longus and the flexor carpi radialis	M. flexor digitorum superficialis	C8, Th1

C: Cervical vertebra; CV: Conception vessel; L: Lumbar vertebra; LI: Large intestine; LR: Liver; PC: Pericardium; S: Sacral vertebra; SP: Spleen; ST: Stomach; Th: Thoracic vertebra.

Table 2. Overview of the study visits.

	Screening visit	**Base line visit**	**Month**				**Follow-up after 4 months of treatment**	**Follow-up 4 months after last treatment**
			1st	2nd	3rd	4th		
Anthropometry: Body composition (weight, height, waist circumference, hip circumference), FG/acne, blood pressure	X						X	X
Menstrual cycle diary	X	X	X	X	X	X	X	X
Questionnaires: EQ-5D, SF36, PCOSQ, CPRS-SA*, Zung SAS#, Zung SDS#, IPAQ, TFEQ-R21, QEWP-R*	X						X	X
Transvaginal ultrasound		X					X	X
Metabolic measures: Fasting blood samples for glucose, insulin, HbA1c, c-peptide, OGTT. Adipokines, lipid profile (LDL, HDL, NEFA) and inflammatory markers		X					X	X
DXA		X					X	X
Endocrine measures: Fasting blood samples for sex steroids, SHBG, LH, FSH, AMH, prolactin, TSH, T4		X					X	X
Tissue and whole blood collection		X					X	X

Antimüllerian hormone (AMH); dual energy x-ray absorptiometry (DXA); EuroQol-5 dimension (EQ-5D); Ferriman–Gallwey score (FG); follicle stimulating hormone (FSH); high density lipoprotein (HDL); hsCRP, International Physical Activity Questionnaire (IPAQ); low density lipoprotein (LDL), luteinizing hormone (LH); non-esterified fatty acids (NEFA); norepinephinre (NE), oral glucose tolerance test (OGTT); polycystic ovary syndrome questionnaire (PCOSQ); Questionnaire of Eating and Weight Patterns-Revised (QEWP-R); sex hormone binding globulin

(SHBG); short form-36 (SF36); Three-Factor Eating Questionnaire (TFEQ-R21); thyroid stimulating hormone (TSH); thyroxine (T4); Zung Self-Rating Anxiety Scale (Zung SAS); Zung Self-Rating Depression Scale (Zung SDS).

* only in Sweden and # only in China.

SPIRIT
STANDARD PROTOCOL ITEMS: RECOMMENDATIONS FOR INTERVENTIONAL TRIALS

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	Page 1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	Page 16
	2b	All items from the World Health Organization Trial Registration Data Set	N/A
Protocol version	3	Date and version identifier	Protocol version: Page 16
Funding	4	Sources and types of financial, material, and other support	See page 16
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	See page 17, author contributions for 5a-d.
	5b	Name and contact information for the trial sponsor	
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	
Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	
	6b	Explanation for choice of comparators	Page 4-5
Objectives	7	Specific objectives or hypotheses	Page 5
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	Page 5-6

Methods: Participants, interventions, and outcomes

Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained Page 5
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists) Page 9-10
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered Page 10-12
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease) Page 10-12
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests) Page 10-12
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial Page 10-12
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended Page 7-8
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure) Table 2
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations Page 14
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size Page 9

Methods: Assignment of interventions (for controlled trials)

Allocation:		
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions Page 5-6

Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned Page 5-6
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions Page 5-6
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how Page 5-6
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial Page 5-6
Methods: Data collection, management, and analysis		
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol Page 5-6
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols Page 5-6
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol Page 5-6
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol page 14-15
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses) Page 14-15
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation) Page 14-15
Methods: Monitoring		
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed Page 15-16

	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial Page 14
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct Page 8-9
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor N/A
Ethics and dissemination		See page 16
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions
	31b	Authorship eligibility guidelines and any intended use of professional writers
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code

Appendices

Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates See appendix
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable See appendix

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.