



Liraglutide in Polycystic Ovary Syndrome - Effects on ovarian dysfunction and thrombotic potential

PhD thesis

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PREFACE

This thesis is written based on research conducted at the Department of Obstetrics and Gynecology and the Department of Internal Medicine, Endocrine Unit, Herlev Gentofte Hospital, between August 2013 and November 2016.

During the past three years I have been enrolled at the Faculty of Health and Medical Sciences, University of Copenhagen, who also supported me with a 3-year Faculty PhD Scholarship. The Department of Obstetrics and Gynecology, has supported my studies, both financially and with clinical as well as office facilities.

The LIPT study, on which's results this thesis is based, was initiated by Caroline Kistorp, Jens Faber and Sven Skouby and funded by a grant from Novo Nordisk A/S. The study was also supported by the Danish Toyota Foundation. None of the funding sources influenced the study design, data collection, data management, data analyses or manuscript writing, but Novo Nordisk had access to Paper II and Paper III four weeks prior to submission.

ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
AFC	Antral follicle count
AMH	Anti-Müllerian hormone
BMI	Body mass index
CI	Confidence interval
COC	Combined oral contraceptives
CVD	Cardiovascular disease
DM2	Type 2 diabetes
ETP	Endogenous thrombin potential
FAI	Free androgen index
FSH	Follicle stimulating hormone
GLP-1	Glucagon-like peptide 1
HbA1c	Glycosylated hemoglobin
HOMA-IR/HOMA2-IR	Homeostasis model assessment-estimated insulin resistance
HDL	High-density lipoprotein
hsCRP	High sensitivity c-reactive protein
ICC	Intraclass correlation coefficient
IUD	Intrauterine device
LDL	Low-density lipoprotein
LH	Luteinizing hormone
MRI	Magnetic resonance imaging
PAI-1	Plasminogen activator inhibitor 1
PCOS	Polycystic ovary syndrome
RCT	Randomized clinical trial
SD	Standard deviation
SHBG	Sex hormone binding globulin
TGT	Thrombin generation test
TVUS	Transvaginal ultrasound
VTE	Venous thromboembolism
vWF	von Willebrand factor

INTRODUCTION

Polycystic ovary syndrome (PCOS) is a complex endocrine disorder in women of fertile age, first described in the literature by Stein and Leventhal in 1935 (1). Approximately 10% of women of reproductive age have PCOS (2) and broad diagnostic criteria lead to a highly heterogeneous PCOS population, encompassing both mild and severe phenotypes. The pathophysiology of the syndrome involves an ovarian and a metabolic dysfunction, intertwined.

The ovarian dysfunction is seen as hyperandrogenism, oligo-/anovulation and polycystic ovarian morphology (3). Two-dimensional transvaginal ultrasound (2D TVUS) is traditionally used for evaluation of ovarian morphology. The novel 3D TVUS has obvious advantages when examining ovarian morphology, e.g. higher reliability (4), but no comprehensive comparison of the two modalities exist and it is not known whether they give similar estimates and can be used interchangeably in a PCOS population.

The metabolic dysfunction of the syndrome mainly consists of insulin resistance and abdominal obesity and PCOS is linked to the metabolic syndrome (5), venous thromboembolism (VTE) (6) and cardiovascular disease (CVD) in later life (7,8). The link between PCOS and CVD is most likely explained by a systemic low-grade inflammation and endothelial dysfunction accompanying the obesity and insulin resistance.

Obesity affects both reproductive and metabolic health negatively and weight control is essential in PCOS treatment. It is well known that even a minor weight loss can have a major impact, ameliorating the reproductive symptoms and lessening the metabolic burden. Apart from lifestyle intervention (9) and metformin (10) there are hardly any noninvasive treatments, altering the body weight and insulin resistance and seemingly no drug exists to efficiently treat the metabolic, inflammatory component and reduce CVD and VTE risk markers. Losing weight can be challenging and achieving a permanent weight loss even more difficult. Many women fail to lose weight or quickly regain the weight lost and therefore it is of great importance to develop pharmacologic interventions to be used alongside with lifestyle intervention in women with PCOS.

Over the last decade glucagon-like peptide-1 (GLP-1) analogs have proven successful in promoting weight loss, improving glycemic control and reducing low-grade inflammation in patients with diabetes type 2 (DM2) and obesity (11–13). Currently, the role of GLP-1 analogs in treating PCOS is being mapped out.

With this thesis we aimed to elucidate the role of 3D TVUS in PCOS research and to add to the knowledge on GLP-1 analog intervention in PCOS. The following research questions were raised:

- Are estimates of ovarian volume and antral follicle count (AFC) from 3D TVUS comparable to estimates obtained by 2D TVUS and by MRI in a PCOS population?
- Does liraglutide improve the ovarian dysfunction as measured by menstrual regularity, levels of androgens and anti-Müllerian hormone (AMH) and ovarian morphology in an overweight PCOS population?
- Does liraglutide intervention affect markers of thrombin generation, endothelial dysfunction and low-grade inflammation in an overweight PCOS population?

To answer these questions a double-blind, randomized, placebo-controlled trial, the LIPT study, was conducted. The study was run as collaboration between the Department of Obstetrics and Gynecology and the Endocrine Unit of the Department of Internal Medicine, Herlev Gentofte Hospital.

This PhD thesis is based on results presented in two of the LIPT study papers (Paper II and III) as well as on results from a study, using baseline data from the LIPT study, validating 3D TVUS against 2D TVUS and MRI, with regard to ovarian morphology (Paper I).

SCIENTIFIC PAPERS INCLUDED IN THE THESIS

I

Nylander M, Frøssing S, Bjerre AH, Chabanova E, Clausen HV, Faber J and Skouby SO.

Ovarian morphology in polycystic ovarian syndrome: Estimates from 2D and 3D ultrasound and magnetic resonance imaging and their correlation to anti-Müllerian hormone.

Acta Radiol, November 9, 2016, doi:10.1177/0284185116676656

II

Nylander M, Frøssing S, Kistorp C, Clausen HV, Faber J and Skouby SO.

Liraglutide in Polycystic Ovary Syndrome: A double-blind, placebo-controlled randomized clinical trial on bleeding ratio, ovarian morphology, Anti-Müllerian hormone and sex steroid levels

Submitted to Reproductive BioMedicine Online

III

Nylander M, Frøssing S, Kistorp C, Faber J and Skouby SO.

Liraglutide in Polycystic Ovary Syndrome: A double-blind randomized clinical trial investigating effects on thrombogenic potential: Thrombin generation, fibrinolysis and low-grade inflammation

Submitted to Metabolism

BACKGROUND

Diagnosis

Polycystic ovary syndrome is the most prevalent endocrine disorder in women of reproductive age.

Depending on the diagnostic criteria used and the population studied the reported prevalence is 6-10% (2).

The syndrome is an exclusion diagnosis based on minimum two out of the three Rotterdam criteria: Oligo/-anovulation, hyperandrogenism and polycystic ovaries (3).

Polycystic ovarian morphology

Polycystic ovarian morphology is evaluated using 2D TVUS and defined as ≥ 12 antral follicles (2-9 mm) and/or ovarian volume > 10 ml in at least one ovary (3). When using newer, improved scanners, operating at higher frequencies (≥ 8 MHz) the threshold of ≥ 12 antral follicles artificially increases the prevalence of polycystic ovarian morphology (14). This ultimately generates a larger and even more heterogeneous PCOS population, as the true PCOS cases are diluted among women with other diagnoses, e.g. hypothalamic amenorrhea. The issue is particularly evident in younger women where polycystic ovarian morphology is common. Recently Lauritsen *et al.* proposed age-adjusted AFC thresholds as they found 68% of women < 30 years to have AFC ≥ 12 (14). In 2014 the *Androgen Excess and PCOS Society* proposed new AFC threshold (≥ 25), but unchanged volume threshold (15), whereas the Rotterdam criteria not yet are revised.

Despite the widespread use of 2D TVUS the reliability and validity of ovarian morphology measurements in PCOS populations are scarcely investigated. Lujan *et al.* found moderate inter-observer reliability for volume estimates, but poor inter-observer reliability for AFC in PCOS (16). In non-PCOS populations the inter-observer reliability is higher with 3D- than 2D TVUS (4,17). Reliability is one thing, validity another. 3D TVUS has never been systematically validated against 2D TVUS or against a proper gold standard, in a PCOS population. Validation of ovarian estimates from TVUS against the true morphology is challenging since oophorectomy in young women is remarkably rare (18,19). Cavalieri's principle used on MRI data is an appropriate gold standard in volume estimation (20) and MRI has previously been proposed as first choice for assessing ovarian morphology in research settings (21). With high reliability, superb soft-tissue resolution and no radiation risk T2-weighted MRI is an appropriate gold standard for validation of both ovarian volume and AFC estimates from 3D TVUS.

It has long been discussed whether serum AMH, which is highly correlated with AFC, preferably should be used as a marker of polycystic ovarian morphology (14,15). However, problems with diversity between AMH assays and in finding a universally accepted method as well as suitable age-specific thresholds have so far delayed this approach.

Pathophysiology

Despite an abundance of research the etiology and pathophysiology of PCOS are still not completely identified. Nevertheless, it is clear that the syndrome has a multifactorial etiology, complex pathophysiology (Figure 1) and heterogeneous clinical presentation. The Rotterdam criteria primarily embody the ovarian dysfunction, but over the last decades it has become evident that PCOS also holds a metabolic dysfunction.

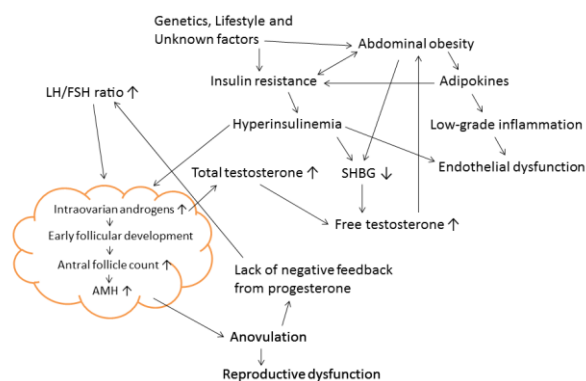


Figure 1. PCOS pathophysiology

Ovarian dysfunction

In PCOS both the folliculogenesis and the ovarian androgen production are altered.

The normal folliculogenesis

The folliculogenesis is a complex biological process. It is coordinated by numerous auto-, para- and endocrine factors, e.g. gonadotropins, sex steroids and AMH, where the relationships between the actors vary in the different stages. In brief, in the presence of follicle stimulating hormone (FSH), androgens, among other factors, stimulate the recruitment of primordial follicles as well as the growth of pre-antral follicles (22). As follicles grow the effect of androgens decline and further development becomes gonadotropin-dependent; FSH stimulates the expression of the aromatase, which leads to rising estradiol levels and further follicular growth and maturation (22). One of the late antral follicles becomes dominant, suppressing the others through estradiol secretion and ultimately proceed to ovulation.

Secreted from the granulosa cells of pre-antral and early antral follicles the glycoprotein AMH has a negative impact on the folliculogenesis. It hinders the initial recruitment of primordial follicles (22,23) and counteracts the gonadotropin-dependent follicle growth as it reduces the sensitivity to FSH (24) and blocks the ovarian conversion of androgens to estrogens by inhibiting expression of the aromatase (25). In the healthy individual this protective effect of AMH therefore acts as a gate-keeper, preventing premature depletion of follicles (22,23).

Ovarian dysfunction in PCOS – androgen excess and anovulation

Androgens are produced in the gonads and adrenals in response to LH and ACTH, respectively and are, by the 5 α -reductase, converted to the more potent dihydrotestosterone in peripheral tissues (26). Androgen levels are not regulated by negative feedback to the same extent as estradiol levels are, as moderately increased testosterone levels do not suppress pituitary LH secretion (26). Ovarian androgen excess is a main feature of PCOS. In vitro studies have shown increased synthesis of testosterone and its precursors to be an intrinsic feature of PCOS theca cells and to be further exacerbated by LH and insulin (27,28). Low levels of sex hormone binding globulin (SHBG) are characteristic for PCOS and linked to hyperinsulinemia (29) as well as abdominal obesity (30). The increased testosterone production and reduced SHBG synthesis contribute to higher levels of free testosterone - the biologically active fraction of the hormone.

It has been proposed that the high intraovarian androgen levels in PCOS promote excessive primordial follicle recruitment, causing the pool of AMH-secreting follicles to enlarge (22,23). High serum AMH is associated with all of the Rotterdam criteria as well as with the severity of PCOS (31–33) and might not only be a marker of, but also a contributor to, the pathophysiology of the syndrome. Due to the high AFC and an increased AMH production per follicle (34) women with PCOS display elevated levels of AMH. Consequently, the AMH gate-keeper mechanism exceeds its physiological purpose causing “follicular arrest” and anovulation (35).

The cause of increased AMH/follicle-ratio in PCOS remains unknown. There is no solid evidence of a direct effect of androgens on AMH production, even though serum levels of androgens and AMH are highly correlated in PCOS (31–33). Possibly, a vicious circle is present as the inhibitory action of AMH on the aromatase (24,25) contributes to intraovarian androgen excess, which further causes the pool of AMH-secreting follicles to grow. Although serum AMH has been found negatively associated with BMI (31,32), weight loss seems to reduce serum AMH alongside with improvement of menstrual regularity in PCOS (36–38). Moreover, markedly elevated serum AMH is predictive of poor response to weight loss with regard to menstrual regularity (36,38,39). Insulin resistance and compensatory hyperinsulinemia cause increased intraovarian testosterone levels (28,40) and are thereby theoretically linked to AMH. However, serum AMH was not found associated with insulin resistance measured using the euglycemic-hyperinsulinemic clamp in 43 women with PCOS and 35 controls (41) or in studies using the homeostasis model assessment-estimated insulin resistance (HOMA-IR) (31,32,39).

Despite PCOS being classified as a normo-gonadotropic disorder, women with PCOS seem to have an increased amplitude and frequency of the pulsatile LH secretion from the pituitary (42). As a consequence an

elevated LH tone (increased LH/FSH ratio) is commonly seen in PCOS (14,42). The insufficient levels of FSH, accompanied by an ovarian FSH resistance (24), interrupt the folliculogenesis while the increased levels of LH stimulate ovarian androgen secretion (28). Progesterone has been found to have an inhibitory effect on LH secretion in normal menstruating women (43) and the, due to anovulation, lack of corpus luteum in PCOS probably further disorientates the hypothalamic-pituitary unit.

Metabolic dysfunction

Insulin resistance

PCOS is characterized by a peripheral insulin resistance that is independent of, but exaggerated by, obesity (44). The insulin resistance is selective, i.e. metabolic but not mitogenic signaling pathways are blunted and consequently the compensatory hyperinsulinemia causes hyperandrogenism by stimulating ovarian androgen production (28,40) and inhibiting hepatic SHBG secretion (29). A vicious circle develops as the androgen-associated visceral adipose tissue (45) indirectly can induce insulin resistance via adipokine secretion (46). The relationship between androgens and insulin resistance is further supported by the fact that hyperandrogenic PCOS phenotypes have higher prevalence of insulin resistance than normoandrogenic phenotypes (47).

Obesity and low-grade inflammation

Overweight is prevalent in women with PCOS. A meta-analysis found prevalence of overweight (BMI ≥ 25 kg/m²), obesity (BMI ≥ 30 kg/m²) and central obesity in PCOS to be 61%, 49% and 54%, respectively (48). Visceral adipose tissue is an important endocrine organ. In the excess adipose tissue dysfunctional adipocytes and infiltrated, activated macrophages secrete a variety of adipokines (e.g. TNF- α , IL-6, leptin), triggering systemic low-grade inflammation (46). Adipokines alter both metabolic, inflammatory and hemostatic functions and represent a link between obesity and CVD (46).

High sensitivity C-reactive protein (hsCRP) is a dynamic marker of systemic inflammation secreted by hepatocytes as a part of the IL-6-stimulated acute phase response (46). Marginally elevated hsCRP levels are, as a marker of low-grade inflammation, associated with subsequent coronary heart disease, ischemic stroke and cardiovascular mortality in persons without known history of CVD (49). A meta-analysis including 15 age- and BMI matched studies found evidence of increased levels of hsCRP in PCOS (50). Our research group has previously found hsCRP to be correlated with BMI and waist circumference, rather than with Rotterdam criteria in 148 women with PCOS (51).

Cardiovascular comorbidities – venous thromboembolism and arterial disease

Women with PCOS are at a markedly higher risk for VTE than non-PCOS women. In a cohort-study including more than 80 000 women Bird *et al.* found the risk to be 2-fold increased among users of combined oral contraceptives (COC) and 1.5-fold increased among non-users (6). Numbers are not adjusted for BMI, but at study entry the prevalence of obesity was 13% in both groups. Moreover, PCOS is associated with CVD as shown in a recent meta-analysis including more than 100 000 women, where the largest impact was provided by two high-quality studies adjusted for several confounders, including age, BMI and smoking (8). Odds ratios for CVD and coronary heart disease were 1.30 (95%CI 1.09-1.56) and 1.44 (95%CI 1.13-1.84), respectively (8). Since cardiovascular events are infrequent in premenopausal women long-term prospective studies on hard CVD endpoints are challenging. The lack of information regarding important confounders (e.g. BMI, smoking and lifestyle) complicates retrospective studies. As a result there is an abundance of studies investigating markers of CVD risk in PCOS and evidence of clustering of conventional CVD risk markers (e.g. hypertension, dyslipidemia, high waist circumference) as well as biomarkers of low-grade inflammation and impaired endothelial function (7,48,50). Moreover, a recent meta-analysis found slightly increased carotid intima-media thickness, a marker of subclinical atherosclerosis, in women with PCOS compared with controls (52).

Endothelial dysfunction and thrombin generation

The healthy endothelium contributes to regulation of blood flow, hemostasis, fibrinolysis and inflammatory processes via secretion of bioactive substances such as nitric oxide, von Willebrand factor (vWF) and plasminogen activator inhibitor 1 (PAI-1) (53). Obesity, hyperglycemia, insulin resistance, dyslipidemia and low-grade inflammation are all linked to endothelial dysfunction, most likely through oxidative stress and reduced nitric oxide availability (53). Endothelial dysfunction is predictive of CVD and includes e.g. hypercoagulability, hypofibrinolysis and overexpression of adhesion-molecules (46,53). There is a complex interplay between low-grade inflammation and hemostasis, as numerous substances (e.g. vWF, PAI-1, fibrinogen) are both hemostatic factors and acute phase reactants (46).

Endothelial dysfunction as measured by pro-thrombotic vWF and anti-fibrinolytic PAI-1

The glycoprotein vWF is mainly produced by endothelial cells and plays a major role in the primary hemostasis, assisting platelet adhesion and aggregation, i.e. attaching platelets to each other and to the injured vessel wall (54,55). Elevated plasma levels of vWF is a sign of endothelial dysfunction and is linked to increased risk of CVD (54). Several smaller studies report similar levels of vWF in women with and without PCOS (56,57), whereas one of the largest studies so far, found elevated vWF levels in 140 women with PCOS compared with 40 age- and BMI-matched controls (58).

Plasminogen activator inhibitor 1 is mainly produced by endothelial cells, but also by hepatocytes and adipocytes in response to metabolic, inflammatory and hormonal stimuli (55). Inhibiting the pro-fibrinolytic enzymes *tissue plasminogen activator* and *urokinase type activator*, PAI-1 plays a key role in the endogenous fibrinolytic system (55). Elevated plasma levels of PAI-1 indicate endothelial dysfunction and decreased fibrinolytic potential and are predictive of CVD events in individuals without prior CVD (59). A meta-analysis of age- and BMI-matched studies reported elevated PAI-1 activity as well as elevated plasma levels of PAI-1 in women with PCOS compared with controls (50). Our group has previously found increased levels of PAI-1 to be driven by both BMI and insulin resistance in PCOS (51).

Thrombin generation

Thrombin is a central factor in the coagulation cascade with multiple, both pro- and anticoagulant functions, including platelet-activation and conversion of fibrinogen into fibrin (60). In vitro thrombin generation can be assessed with the thrombin generation test (TGT), which measures the global thrombotic capacity, i.e. both pro- and anticoagulant components, rather than isolated coagulation factors or parts of the coagulation cascade (60). In the TGT the thrombin concentration, as a function of time, is presented in a thrombogram, where four parameters usually are assessed: 1) lag-time; 2) peak thrombin concentration; 3) time to peak and 4) area under curve (i.e. endogenous thrombin potential (ETP)) (60).

Increased ETP and peak thrombin concentration are good indicators of a hypercoagulable state, e.g. in COC-users (61). High thrombin generation measured as ETP has been found associated with increased risk of first (62) and recurrent VTE (63). The relationship between thrombin generation and CVD is less clear. In a prospective cohort study, assessing a background population ≥ 65 years, high ETP was found associated with increased risk of ischemic stroke in women, whereas no link between ETP and coronary heart disease was found (64). Also, an association between ETP levels and the degree of coronary atherosclerosis has been reported in patients with stable angina (65).

Thrombin generation appears elevated in PCOS. Glintborg *et al.* found higher ETP and peak thrombin concentration in 90 women with PCOS compared with 35 age- and BMI matched controls, whereas Burchall *et al.* found no statistically significant difference in ETP between PCOS and controls (n=123) (66,67). Using a slightly different TGT, Mendonça-Louzeiro *et al.* found faster thrombin generation but unaltered ETP in women with PCOS (68). In PCOS ETP is correlated to central obesity and low-grade inflammation but not to levels of total testosterone (66,69,70). In a previous study on 148 women with PCOS, our group found higher levels of ETP in overweight and insulin resistant women than in normal-weight women without insulin resistance (69). The difference disappeared after adjusting for either waist circumference or hsCRP levels.

Also in other populations ETP seems mainly driven by central obesity and low-grade inflammation and to a lesser extent by impaired glucose metabolism (71–73).

Treatment options

Depending on the clinical presentation there are different treatment options for women with PCOS, without current pregnancy wish. Lifestyle intervention, promoting weight loss, improves both the ovarian and metabolic function and should be first-line treatment (9). Combined oral contraceptives effectively improve menstrual regularity and reduce hyperandrogenism, but are associated with increased risk of arterial and venous thrombosis (74,75). Hirsutism can to some extent be treated with systemic anti-androgenic therapy such as spironolactone (76) and topical intervention with eflornithine or photoepilation (77). Metformin improves menstrual regularity and slightly affects body composition and androgen levels (10,78), most likely through reduced hyperinsulinemia. Also, inositol, optimizing insulin signaling, have been proposed as a treatment option (79). Moreover, cyclic progestin treatment or a levonorgestrel-containing intrauterine device (IUD) can provide protection from endometrial hyperplasia (80).

Glucagon-like peptide-1 as a novel intervention in PCOS

Glucagon-like peptide-1 (GLP-1) is an incretin hormone secreted by enteroendocrine L-cells, in response to presence of nutrients in the intestinal lumen (82). The GLP-1 receptor is found in numerous tissues, including the human endocrine pancreas as well as primate lung, kidney, heart and gastro-intestinal tract (81). Endogenous GLP-1 stimulates glucose-mediated insulin secretion, inhibits postprandial glucagon secretion, and decelerates gastric emptying (82). Moreover, GLP-1 reduces appetite via a CNS mechanism (82). Long-acting GLP-1 analogs are used in the treatment for DM2 and obesity, where they reduce body weight, HbA1c and markers of low-grade inflammation (11–13).

The first study on GLP-1 analogs in PCOS, published in 2008, found exenatide to improve menstrual frequency and to reduce BMI as well as levels of testosterone (83). Since we undertook this study, several trials have investigated the effect of the GLP-1 analog liraglutide in PCOS, primarily with regard to body weight, glucose metabolism and levels of androgens and blood lipids (84–89). However, none of the studies are blinded or placebo-controlled, most are short-term (84–87,89), some are single-armed (87,88) and others assess liraglutide as an add-on to metformin (85,90).

HYPOTHESES AND OBJECTIVES

With this PhD thesis we aimed to add to the knowledge gaps regarding 1) the use of 3D TVUS in PCOS and 2) the effect of GLP-1 analog intervention in PCOS.

The 3D TVUS has several advantages, which is why we aimed to explore whether this modality could be used in a PCOS population, interchangeable with 2D TVUS. Since PCOS is a prevalent disorder with severe comorbidities we wanted to explore the feasibility of the novel intervention liraglutide in this population, focusing on markers of ovarian function and cardiovascular risk.

We hypothesized that liraglutide intervention in women with PCOS would result in improved ovarian function seen as improved bleeding frequency, decreased levels of androgens and possibly decreased levels of AMH as well as altered ovarian morphology. These effects might be due to weight loss and improved glucose metabolism, i.e. reduced hyperinsulinemia. Moreover, we theorized that liraglutide intervention also would cause reduced low-grade inflammation, thrombin generation and endothelial dysfunction, via weight loss and improved glucose metabolism.

Specific objectives

Study I

Study I is a methodological study with the objective of determining the role of the novel 3D TVUS modality in the assessment of PCOS ovaries. When investigating the effect of liraglutide on ovarian morphology (Study II) a reliable and precise imaging modality, with the possibility of image archiving, would be preferable. Therefore we compared ovarian volume and AFC estimates from 3D TVUS with estimates from the traditional 2D TVUS approach as well as MRI.

Study II

The aim of Study II was to investigate the effect of liraglutide on ovarian function in PCOS. We investigated the effect on bleeding frequency, ovarian morphology (ovarian and stromal volume and AFC) as well as on serum levels of androgens, SHBG and AMH.

Study III

The objective of Study III was to investigate the effect of liraglutide intervention on surrogate markers of pro-thrombosis and low-grade inflammation: Thrombin generation, plasma levels of vWF, PAI-1 and hsCRP, in a PCOS population.

MATERIAL, METHODS AND METHODOLOGICAL CONSIDERATIONS

The LIPT study was a randomized clinical trial conducted at the Department of Obstetrics and Gynecology and the Department of Internal Medicine, Endocrine Unit, Herlev Gentofte Hospital, in March 2014 - December 2015. The study was approved by the Ethics Committee of the Capital Region of Denmark, the Danish Health Authority and the Danish Data Protection Agency, registered at clinicaltrials.gov and monitored by the GCP unit of Copenhagen University Hospitals. The trial was conducted in accordance with the declaration of Helsinki. Oral and written consent were obtained for each participant prior to inclusion.

Study I – Ovarian morphology

This study was cross-sectional, using baseline data from the LIPT study, comparing ovarian morphology from 2D-, 3D TVUS and MRI. At baseline visit, 2D-, 3D TVUS, MRI scan and blood sampling were performed within four hours. Blood was drawn between 8-10 AM, centrifuged and stored at -80°C for later analysis of serum AMH. The assay used for analysis of serum AMH is described in Paper I. Every other woman contributed with estimates from the right ovary and every other with estimates from the left. If the ovary was unidentified or had a larger follicle or a corpus luteum the contralateral ovary was chosen for analysis. All follicles measuring 2-9 mm were included in the AFC. The ultrasound investigator was blinded to MRI results and vice versa. The 2D TVUS estimates and 3D TVUS data were obtained in the same session. 3D data were evaluated at least two weeks after the visit and analysis was blinded to the 2D results. To estimate intra-observer reliability ten randomly selected 3D TVUS datasets and ten randomly selected MRI datasets were re-evaluated at least two months after first analysis. The same datasets were evaluated for inter-observer reliability, where the TVUS observer assessed the MRI data and vice versa.

Transvaginal ultrasound was performed by a single observer (MN) in a standardized way using a Voluson E6 scanner with a 5-9 MHz transducer (GE Healthcare, Chicago, IL, USA). From 2D TVUS ovarian volume was calculated using the maximum longitudinal, transverse and antero-posterior diameter and the formula of an ellipsoid. The AFC was obtained from sweeping motions in two planes perpendicular to each other, where the mean of the two counts was recorded. The 3D data were post-processed using the 4DView software (GE Healthcare, Chicago, IL, USA). A 3D model was created by outlining the ovarian contour in 12 sections, on an image rotating around the Y-axis, using the VOCAL-tool (Figure 2). The software automatically calculates the volume inside the marked contour. The AFC was determined with the SonoAVC-tool, which color-codes hypo-echogenic areas (follicles) in the 3D dataset. Images were manually post-processed, e.g. if follicles were missed out or if multiple follicles were perceived as one it was corrected.

A T2-weighted MRI was performed, by a single operator with an Achieva 3.0 T MR Imaging System (Philips Medical systems, Best, the Netherlands) and a sense cardiac coil. Images of the lower abdomen and pelvis,

constructed from 2 mm thick sections, were analyzed by a single investigator on a Philips ViewForum workstation, using the “Segmentation tool” in the “Volume analysis”. The ovarian contour was outlined in each section and the volume was automatically calculated from the area and thickness of the sections (Cavalieri’s principle). The AFC was determined by scrolling through the 2-mm sections.

The primary outcome was mean difference between ovarian volume estimates from 2D- and 3D TVUS. Secondary outcomes included mean difference between ovarian volume estimates from 2D TVUS and MRI and between 3D TVUS and MRI as well as mean difference in AFC between the three modalities. Moreover, the correlations between AFC from the three modalities and serum AMH were calculated.

Based on a previous study, comparing 2D TVUS and MRI a sample size calculation was done (21). With 80% power and a significance level of 0.05 we needed 60 ovaries to find a 1.7 ml difference in ovarian volume between 2D- and 3D TVUS. We considered 1.7 ml a clinically relevant size. Paired t-tests and Bland Altman statistics were used for comparison of volume and AFC estimates from different modalities, as the differences were normally distributed. Correlations between serum AMH and AFC were determined with Spearman’s correlation coefficient. Moreover, a multiple regression analysis with AFC as dependent variable and age, BMI and serum AMH as covariates was performed. Intra- and inter-observer reliability was calculated for 3D TVUS and MRI using intraclass correlation coefficients (ICC 2,1) from a two-way random effects ANOVA with absolute agreement. SAS® Enterprise Guide 7.1, SAS 9.4 (SAS Institute Inc., Cary, NC, USA) was used for statistical analyses and two-sided p-values ≤ 0.05 were considered significant. IBM SPSS Statistics 22 (IBM, Armonk, NY, USA) was used for calculation of ICC.

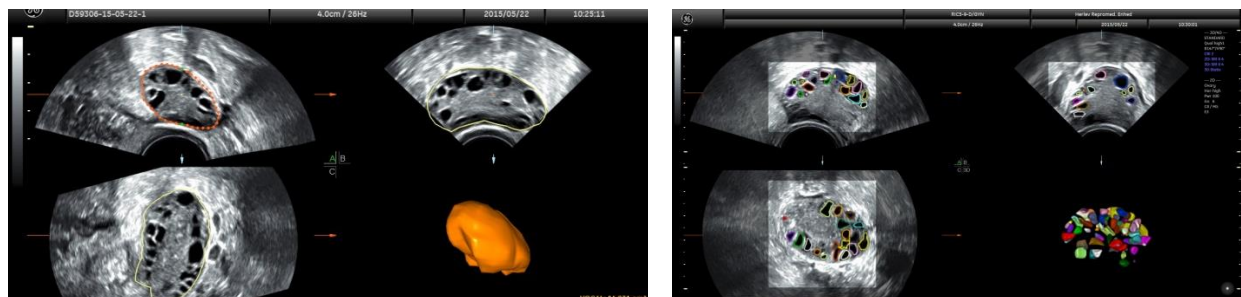


Figure 2. 3D transvaginal ultrasound. VOCAL-tool (left) and SonoAVC-tool (right).

Considerations – Study I

Validity

The concurrent criterion validity refers to the degree to which an estimate correlates with another estimate of the same object measured at the same time. We aimed to test the validity of ovarian morphology estimates from 3D TVUS by comparing them to the “traditional” standard 2D TVUS and the “gold standard”

MRI. Moreover, the correlation between AFC from 3D TVUS and serum AMH was determined as a third “biochemical” validation.

Blinding

Blinding is essential for the determination of both reliability and validity, which is why one investigator performed TVUS and another analyzed the MRI images. 2D TVUS estimates were measured during the scanning and 3D data were assessed at least two weeks later, to ensure blinding.

Statistics

Due to dependency of volume and AFC estimates from the ovaries within one woman, studying both ovaries of each participant would result in an incorrect doubling of the sample size. With dependent observations the variance and SD decrease, which might cause inaccurate statistic conclusions. Therefore, we alternated between using estimates from the right and left ovary. When calculating intra- and inter-observer reliability for 3D TVUS and MRI we used the intra-class correlation coefficient (ICC). When used for reliability measures the Bland Altman statistics determine the between-observer variation, but take no account of the between-subject variation, whereas the ICC addresses both.

Study II and Study III – The LIPT study

In total, 72 women were randomized, in a 2:1 ratio, to 26 weeks of intervention with liraglutide or placebo. Study medication was administered subcutaneously as 1.8 mg/day, starting with 0.6 mg/day the first week and escalating through 1.2 mg/day, for the second week. Novo Nordisk A/S, Denmark provided a randomization list and study drug in prefilled pens, identically labeled and packed. Participants and investigators were blinded as a secretary, otherwise not involved in the study, handled the randomization list in privacy and instructed the investigators in which packages of medication to be handed out to each participant.

Eligible women were ≥ 18 years, premenopausal, with PCOS according to Rotterdam criteria, BMI $\geq 25 \text{ kg/m}^2$ and/or insulin resistance (fasting plasma C-peptide $> 600 \text{ pmol/l}$) and without diabetes, hypertension, overt inflammatory disease or cancer (within the last five years). The use of hormonal contraceptives within the last six weeks, antidiabetic/-androgenic drugs within the last three months and the use of drugs known to affect the hemostatic and cardiovascular systems led to exclusion. In- and exclusion criteria are described in detail elsewhere (91).

Each woman attended a screening-, baseline-, safety- and follow-up visit. At baseline- and follow-up visits the women met after overnight fasting. Anthropometric measures and Ferriman-Gallway score were

evaluated, blood tests and an oral glucose tolerance test were obtained and a full body DXA scan, an abdominal MRI scan and a 3D TVUS scan were performed. At baseline the number of bleeding episodes during the last six months was recorded. During the study participants kept a bleeding diary, registered compliance daily and were encouraged to contact the investigators if they experienced adverse effects. Bleeding ratio at baseline was defined as number of bleedings during the last six months divided by six and bleeding ratio at follow-up as number of bleedings during the study period divided by months in the study. As pregnancy was an exclusion criterion insertion of a copper IUD was mandatory for the participants. In case of any contraindication the participant was instructed in concomitant use of diaphragm and condom, in accordance with guidelines from the Danish Health Authority.

For Study II outcomes included between-group difference in change from baseline to follow-up in bleeding ratio, ovarian volume, stromal volume, AFC and levels of androgens, SHBG and AMH. In Study III the primary outcome was between-group difference in change in ETP, assessed by TGT, between baseline and follow-up. Secondary outcomes in Study III included between-group difference in change from baseline to follow-up in other TGT parameters: peak thrombin concentration, lag-time and time to peak as well as levels of vWF, PAI-1 and hsCRP. Metabolic markers: levels of fasting glucose, fasting insulin, HbA1c, cholesterol and triglycerides were also assessed. Assays are described in detail in Paper II and Paper III.

Based on a standard deviation of 130 units of ETP (nmol/min) obtained from in-house data the sample size calculation declared 63 women needed for an 80% power to find a difference in effect size of 100 nmol/min. To allow for dropouts 72 women were randomized. Power calculation for bleeding ratio is reported in Paper II. Non-normally distributed data were subject to logarithmic transformation. Between-group differences at baseline were assessed using unpaired t-test, Mann Whitney U test, χ^2 or Fisher's exact test, as appropriate. Within-group difference from baseline to follow-up was estimated using a paired t-test (per protocol) and between-group difference in effect size was calculated using a linear mixed model with repeated measurements and maximum likelihood. Here, missing data are assumed to be missing at random. Fisher's exact test was used for comparison of adverse effects between groups. In analyses on hsCRP levels estimates >10 mg/L, indicating infection, were excluded. For TGT measures predefined subgroup analyses were performed for the Rotterdam phenotypes and metabolic subgroups ($\text{HOMA2} < 2.3 + \text{BMI} < 25$; $\text{HOMA2} < 2.3 + \text{BMI} \geq 25$; $\text{HOMA2} \geq 2.3 + \text{BMI} < 25$ and $\text{HOMA2} \geq 2.3 + \text{BMI} \geq 25$). Due to the size of the groups non-parametric statistics (Mann Whitney U test) with Bonferroni correction were used for subgroup analyses. The HOMA2-IR was calculated as described in Paper III. Associations between ETP and anthropometric, metabolic and endocrine covariates were assessed using Pearson's and Spearman's correlations and multiple linear regression analyses. The change in bleeding ratio and change in AMH in

relation to change in anthropometric, endocrine, metabolic and ovarian morphology variables as well as to bleeding ratio at baseline were analyzed using Pearson's and Spearman's correlation coefficients and multiple linear regression. P-value ≤ 0.05 was considered significant. Statistical analyses were performed with SAS® Enterprise Guide 7.1, SAS 9.4, SAS Institute Inc., Cary, NC, USA.

Considerations – Study II and Study III

The randomized trial

The RCT design ranks high in the evidence hierarchy, only exceeded by the meta-analysis of RCTs. Randomization eliminates the risk of selection bias and ensures that participants, at baseline, are comparable with regard to known and unknown confounders. Randomization was performed in a 2:1 ratio as we assumed women to be more motivated to enroll if there was a higher chance of receiving liraglutide intervention. A placebo group is important to account for the “effect of expectation” with regard to the more “subjective” outcomes as bleeding pattern and adverse effects but also with regard to a weight loss and related alterations in biomarkers and ovarian morphology. Moreover, the placebo group serves as a control, ensuring reliability of the methods used. Double-blinding ensures unbiased sources of data (both participants and investigators) for evaluation of outcomes.

Recruitment strategy

To ensure external validity we aimed to include an unselected population of women with PCOS. Apart from enrolling women from our outpatient PCOS clinic and the endocrine outpatient clinic we advertised at universities and other institutions in Copenhagen, as well as in the local area. Our recruitment strategy included advertising on the internet: the Herlev Hospital homepage (www.herlevhospital.dk), the intranet of RegionH as well as www.sundhed.dk and www.forsogsperson.dk. Since the potential participants were relatively young and most often not in regular contact with a primary care physician or a specialist, recruiting via social media seemed applicable and a Facebook page was created (www.facebook.com/PCOSkliniskforsoeg). Moreover, we sent information and posters to general practitioners in the local area and private gynecologists in the RegionH, contacted the Danish PCOS patient association and invited participants from a previous study conducted by our group (69).

Thrombin generation assay

When planning this study the sparse evidence available suggested ETP, measured by TGT, to be a proper measure of VTE risk and possibly also a measure of atherosclerosis and CVD risk (63,65). The idea of using measures of both pro-thrombosis (vWF and ETP), hypo-fibrinolysis (PAI-1) and low-grade inflammation (hsCRP) seemed appropriate. However, papers on TGT published since we initiated the LIPT study have caused basis for confusion, reporting none, positive or negative associations between ETP and CVD (92).

Nevertheless, ETP still seems a good indicator of hypercoagulability and VTE risk and an interesting and relevant marker in a population with increased VTE risk.

Statistics

Initially we planned on using an unpaired t-test on the intention-to-treat population when calculating the between-group effect size. However, a mixed model with maximum likelihood is a more sophisticated way of analyzing repeated measurements and this statistic approach was chosen instead. An ETP effect size of 100 nmol/min was reinforced by results from a previous study where a similar decrease in ETP was seen together with a 5% decrease in BMI (93).

RESULTS

Study I – Ovarian morphology

Of 72 women randomized in the LIPT study three had claustrophobia and three did not fit into the MRI scanner (Figure 3). Sixty-six paired observations were used for comparison of ovarian morphology estimates from 2D-, 3D TVUS and MRI. Due to technical problems 3D data on AFC were missing in four cases. In the 66 women median (range) age was 29 years (19-44) and mean (SD) BMI 32.7 (4.5) kg/m². Mean (SD) ovarian volume was 9.4 (3.6) ml, 10.9 (3.7) ml and 10.7 (4.1) ml from 2D-, 3D and MRI, respectively. Corresponding numbers for median (range) AFC were 26.5 (4-64), 29 (7-97) and 29 (4-98).

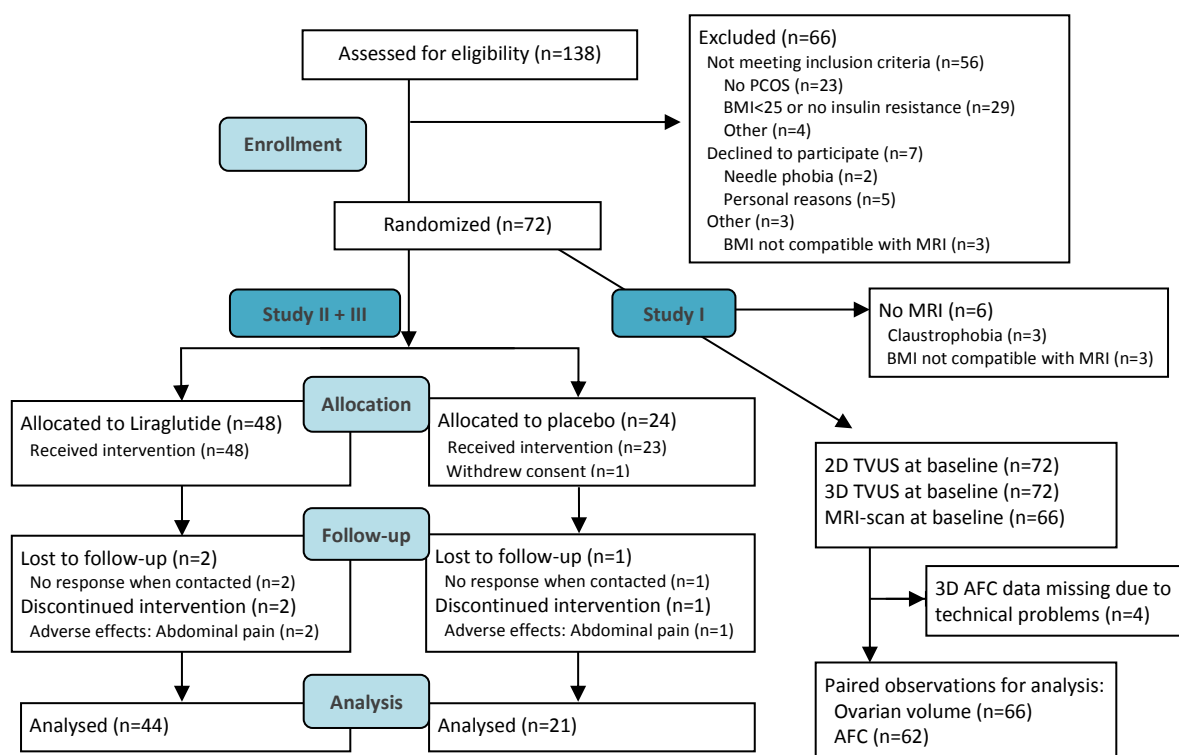


Figure 3. Patient flow in Study I, II and III

Trumpet-shaped Bland Altman plots for volume- and AFC comparisons indicate higher differences with higher estimates (Figure 4), which is why mean differences are presented as percentages. We found estimates of ovarian volume from 2D TVUS to be 15% (95%CI 10-19%, $p<0.001$) and 12% (95%CI 6-17%, $p<0.001$) smaller than estimates from 3D TVUS and MRI, respectively.

With regard to AFC, 2D TVUS estimates were 18% (13-23%, $p<0.001$) smaller than estimates from 3D TVUS and 16% (6-25%, $p<0.005$) smaller than estimates from MRI. There were no significant differences between 3D TVUS and MRI with regard to volume or AFC estimates.

Polycystic ovarian morphology (according to Rotterdam criteria) was seen in 60/66 (91%) and 63/66 (95%) of the women, with 2D- and 3D TVUS, respectively.

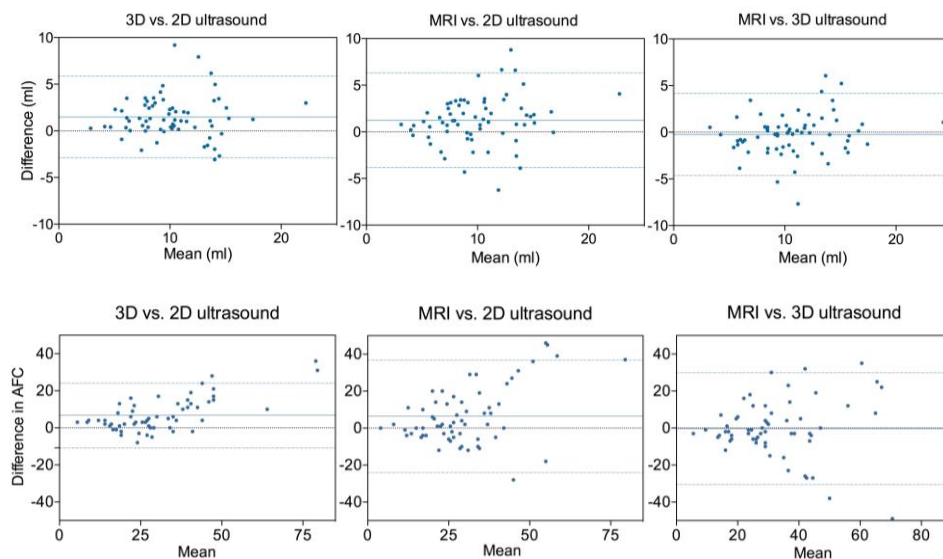


Figure 4. Bland Altman plots showing mean differences (full line) and limits of agreement (dashed lines) for comparisons of volume (upper panel) and AFC (lower panel) estimates from 3D TVUS, 2D TVUS and MRI. AFC – antral follicle count; MRI – magnetic resonance imaging. Inspired by Fig. 3 and 4 in Nylander et al., Acta Radiol doi:10.1177/0284185116676656

Relation between serum AMH and AFC

Serum AMH correlated well with AFC from all three modalities; Spearman's r was 0.67, 0.78, and 0.70 for AFC from 2D-, 3D TVUS and MRI, respectively ($p<0.001$ for all).

Reliability

For volume estimates intra-observer ICCs (95%CI) for 3D TVUS and MRI were 0.957 (0.888-0.984) and 0.952 (0.862-0.982), respectively. Corresponding numbers for AFC were 0.987 (0.966-0.995) and 0.691 (0.026-0.897). Inter-observer ICCs for volume estimates from 3D TVUS and MRI were 0.939 (0.769-0.980) and 0.829 (0.431-0.940), respectively, and corresponding numbers for AFC were 0.845 (-0.028-0.964) and 0.797 (-0.041-0.948).

Study II and Study III - the LIPT study

Of 138 women who were screened 72 were randomized and 65 completed the study (Figure 3). Of the randomized women 73.6% were recruited from social media or other internet sources, 13.9% from outpatient clinics, general physicians or private gynecologists, 2.8% from a previous study (69) and 9.7% from advertising in the local area and at teaching institutions. There were no statistically significant differences in baseline characteristics between groups, but trends towards higher age ($p=0.11$), lower vWF- ($p=0.06$) and hsCRP levels ($p=0.10$) and shorter lag-time ($p=0.08$) in the liraglutide group (Table 1-4). Dropout ratio was 4/48 (8.3%) in the liraglutide group, 3/24 (12.5%) in the placebo group and 7/72 (9.7%) overall. In the placebo group one woman withdrew her consent at the day of randomization. Otherwise, reasons for dropout were similar in the groups: abdominal pain (4.2%) and lost to follow-up (4.2%). Dropouts did not differ from women who completed the study, with regard to age, BMI and bleeding ratio. Median (p25-p75) compliance was 96% (89-99%) and 93% (87-98%) in the liraglutide and placebo group, respectively (difference between groups, $p=0.70$). Adverse effects are presented in Paper III.

Table 1. Baseline characteristics of the 72 women randomized in Study II and Study III.			
	LIRAGLUTIDE (n=48)	PLACEBO (n=24)	p
Age (years)	31.4 (24.6-35.6)	26.2 (24.8-31.5)	0.11
BMI (kg/m ²)	33.3 (5.1)	33.3 (4.6)	0.98
Waist (cm)	102.6 (10.8)	102.6 (11.1)	0.99
Smoking (<10 cigarettes/day)	18.8% (9)	33.3% (8)	0.24
Ethnicity			0.68
	Caucasian 93.8% (45)	91.7% (22)	
	Asian 2.1% (1)	0	
	African 2.1% (1)	0	
	Middle east 2.1% (1)	8.3% (2)	
Rotterdam phenotype			0.54
	O/A + HA + PCO 39.6% (19)	45.8% (11)	
	O/A + HA 2.1% (1)	8.3% (2)	
	HA + PCO 22.9% (11)	16.7% (4)	
	O/A + PCO 35.4% (17)	29.2% (7)	
Amenorrhea / Oligomenorrhea / Regular menstruation	23%/60%/17% (8/29/11)	21%/58%/21% (5/14/5)	
Ferriman Gallway score	7.0 (3.0-11.5)	7.0 (2.5-12.5)	0.90
GFR (ml/min/1.73 m ²)	112.8 (13.3)	117.5 (9.7)	0.12
Data presented as mean (SD), median (p-25-p75) or percentages (n). ¹ one missing, ² two missing, ⁴ four missing. Between-group differences at baseline were determined by un-paired t-test, Mann Whitney U-test, Chi ² -test or Fisher exact test as appropriate. O/A - oligo-/amenorrhea, HA - hyperandrogenism, PCO - polycystic ovarian morphology, GFR - glomerular filtration rate.			

Study II - Ovarian dysfunction

Bleeding ratio increased in both groups and at 26-week follow-up bleeding ratio was improved by 0.14 (95%CI 0.03-0.24, $p<0.05$) in the liraglutide group as compared with placebo (Table 2). Excluding women with regular menstruation at baseline (n=16) from the analyses did not alter the results. In the liraglutide

group ovarian and stromal volume was reduced by 2.0 ml (95%CI 0.9-3.1, $p<0.001$) and 1.9 ml (95%CI 0.8-3.1, $p<0.001$), respectively and there was a trend towards reduction as compared with placebo (Figure 5). Moreover, levels of SHBG increased by 18% (95%CI 3-36, $p<0.05$) and free androgen index decreased by 24% (95%CI 4-39, $p<0.05$) in the liraglutide group as compared with placebo.

Relationship between change in bleeding ratio and anthropometric, metabolic and endocrine parameters

The change in bleeding ratio correlated with the reduction in BMI ($r=-0.28$, $p<0.05$) and with baseline bleeding ratio ($r=-0.66$, $p<0.001$), but not with change in: waist, testosterone, AMH, gonadotropins, fasting glucose, insulin, HOMA2-IR, ovarian volume or AFC (data not shown). In a multiple linear regression analysis with change in bleeding ratio as dependent variable, both bleeding ratio at baseline ($p<0.001$) and study drug ($p<0.05$) were significant covariates (model: $R^2=0.49$, $p<0.01$). When adjusting for change in BMI, but not when adjusting for change in HOMA2-IR, the covariate study drug became non-significant ($p=0.26$ and $p<0.05$, respectively).

The decrease in serum AMH was correlated with change in total testosterone ($r=0.47$, $p<0.0001$), free testosterone ($r=0.38$, $p<0.01$) and AFC ($r=0.29$, $p<0.05$). There was no correlation between the change in serum AMH and the change in: BMI, waist circumference, gonadotropins, fasting glucose, fasting insulin or HOMA2-IR (data not shown).

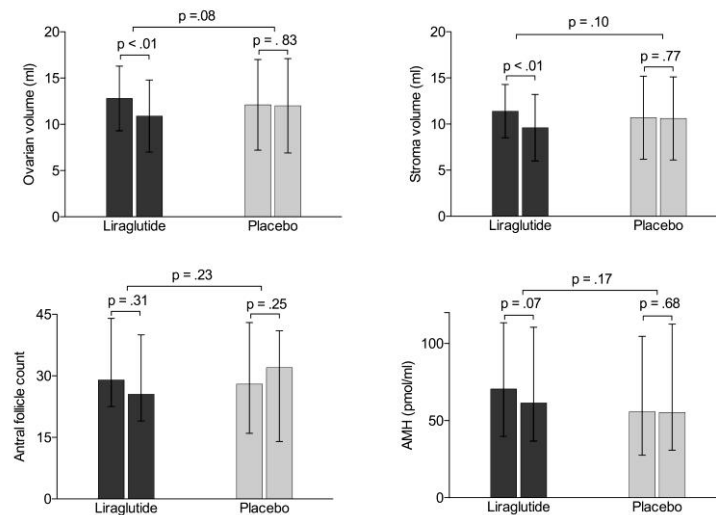


Figure 5. Ovarian morphology markers: Baseline and follow-up values

Data are presented as means (± 1.96 SD) for ovarian volume and stromal volume and as medians (p25-p75) for antral follicle count and serum anti-Müllerian hormone (AMH). P-values for within-group differences from baseline to 26-week follow-up and between-group difference in effect size are displayed. Dark grey columns present liraglutide, light grey columns present placebo.

	LIRAGLUTIDE			PLACEBO			BETWEEN-GROUP			
	Baseline (n=48)	Difference (n=44)	p	Baseline (n=24)	Difference (n=21)	p	Crude	p	Adjusted	p
Bleeding ratio	0.67 (0.33-0.83)	0.28 (0.20-0.36) ²	<.001	0.58 (0.33-0.83)	0.14 (0.02-0.26) ³	0.03	0.14 (0.03-0.24)	0.01	0.13 (0.02-0.24)	0.02
Ovarian volume (ml)	12.8 (3.5) ¹	-2.0 (-3.1- -0.9) ¹	<.001	12.1 (4.9) ¹	-0.2 (-1.7-1.4) ¹	0.83	-1.6 (-3.3-0.1)	0.07	-1.57 (-3.30-0.17)	0.08
Stroma volume (ml)	11.4 (2.9) ⁴	-1.9 (-3.1- -0.8) ²	<.01	10.7 (4.5) ²	-0.2 (-1.7-1.2)	0.77	0.86 (0.71-1.03)^	0.09	0.86 (0.72-1.03)^	0.10
Antral follicle count	29.0 (22.5-44.0) ⁴	-2.0 (-6.0-2.0) ²	0.31	28.0 (16.0-43.0) ²	2.5 (-2.0-7.0)	0.25	0.88 (0.74-1.06)^	0.17	0.90 (0.75-1.08)^	0.23
Follicular volume (ml)	1.4 (0.7) ⁴	-0.2 (-0.4-0.1) ²	0.14	1.4 (0.7) ²	0.0 (-0.2-0.3)	0.71	0.93 (0.71-0.93)^	0.57	0.94 (0.72-1.22)^	0.65
AMH (pmol/ml)	70.5 (39.7-113.4)	-8.4 (-17.4-0.6)	0.07	72.3 (27.5-104.7) ¹	3.5 (-13.9-21.0) ¹	0.68	0.87 (0.72-1.04)^	0.13	0.88 (0.74-1.06)^	0.17
LH (IU/L)	8.0 (5.1-12.9)	-1.7 (-5.9-2.6)	0.43	8.7 (4.5-14.2)	1.0 (-2.7-4.6)	0.59	1.08 (0.73-1.59)^	0.70	1.12 (0.77-1.63)^	0.54
FSH (IU/L)	6.1 (3.8-7.9)	-0.3 (-1.3-0.8)	0.64	5.8 (4.6-6.6)	0.2 (-1.3-1.7)	0.76	0.95 (0.74-1.21)^	0.68	0.95 (0.74-1.22)^	0.69
Estradiol (nmol/L)	0.25 (0.17-0.58)	-0.04 (-0.07-0.14)	0.49	0.24 (0.19-0.39)	0.02 (-0.14-0.11)	0.80	1.01 (0.74-1.39)^	0.94	1.00 (0.73-1.37)^	0.98
Total testo (nmol/L)	1.23 (0.91-1.63)	-0.07 (-0.25-0.10)	0.41	1.35 (0.95-1.93)	0.15 (-0.10-0.39)	0.23	0.88 (0.71-1.09)^	0.26	0.90 (0.73-1.10)^	0.30
Free testo (nmol/L)	0.026 (0.021-0.038)	-0.005 (-0.009- -0.001)	0.01	0.033 (0.023-0.040)	0.004 (-0.003-0.011)	0.22	0.81 (0.65-1.00)^	0.05	0.82 (0.67-1.01)^	0.06
FAI	3.84 (2.78-6.54)	-1.34 (-2.19- -0.48)	<.01	4.95 (3.08-6.32)	0.80 (-0.42-2.01)	0.18	0.74 (0.58-0.95)^	0.02	0.76 (0.61-0.96)^	0.02
Androst. (nmol/L)	6.31 (4.39-7.93) ¹	-0.69 (-1.44-0.06)	0.07	6.29 (4.63-8.84)	0.76 (-0.39-1.92)	0.18	0.85 (0.70-1.04)^	0.11	0.88 (0.73-1.07)^	0.20
SHBG (nmol/L)	31.0 (22.0-44.5)	7.4 (4.1-10.7)	<.001	30.5 (23.0-37.5)	2.0 (-2.9-7.0)	0.40	1.19 (1.02-1.39)^	0.03	1.18 (1.03-1.36)^	0.02
Data at baseline presented as mean (SD) or median (p25-p75) and differences as mean (95% CI) or mean ratio (95% CI) [^] from logarithmic transformed numbers. Between-group differences at baseline were determined by un-paired t-test or Mann Whitney U-test, as appropriate. ¹ = one missing, ² = two missing, ⁴ = four missing and [^] = presented as a ratio. AMH - anti-Müllerian hormone; FAI - Free Androgen Index; FSH - follicle stimulating hormone; LH, luteinizing hormone; SHBG - Sex hormone binding globulin										

Study III - Thrombin generation, endothelial dysfunction and low-grade inflammation

We observed no effect on ETP levels in either group (Table 3, Figure 6). From baseline to follow-up peak thrombin concentration decreased by 16.71 nmol/L (95%CI 2.32-31.11, $p<0.05$), lag-time increased by 0.13 min (95%CI 0.01-0.25, $p<0.05$) and time to peak increased by 0.38 min (95%CI 0.09-0.68, $p<0.05$) in the liraglutide group, with no between-group differences. In the liraglutide group PAI-1 decreased by 12% (95%CI 0-23, $p=0.05$) and there was a trend towards decreased PAI-1 levels when compared with placebo, -16% (95%CI -32-4, $p=0.10$). Moreover, there was a trend towards reduced levels of hsCRP in the liraglutide group, -15% (95%CI -30-3%, $p=0.09$) but no between-group difference. Adjustment for age, BMI and smoking status at baseline did not affect the results (data not shown). Changes in anthropometrics and metabolic markers are presented in Table 4.

Table 3.

Baseline values, within-group and between-group differences for markers of thrombin generation, endothelial dysfunction and low-grade inflammation

	LIRAGLUTIDE		PLACEBO		BETWEEN-GROUP
	Baseline (n=48)	Difference (n=44)	Baseline (n=24)	Difference (n=21)	Crude
ETP ($\mu\text{mol/L} \times \text{min}$)	1796 (332)	-57.6 (-132.3-17.2)	1830 (285)	-8.2 (-98.7-82.3)	-47.3 (-161.9-67.3)
Peak thrombin (nmol/L)	247.3 (41.1)	-16.7 (-31.1- -2.3)*	245.7 (46.5)	-3.3 (-22.9-16.3)	-12.6 (-35.5-10.3)
Time to peak (min)	7.36 (1.11)	0.38 (0.09-0.68)*	7.86 (1.19)	0.12 (-0.30-0.54)	0.25 (-0.25-0.75)
Lag-time (min)	3.33 (2.92-3.67)	0.13 (0.01-0.25)*	3.59 (3.00-3.84)	0.09 (-0.09-0.26)	0.04 (-0.17-0.24)
vWF (% of normal)	99.5 (87.5-122.5)	1.64 (-3.3-6.6)	123.0 (92.0-145.0)	-5.2 (-12.1-1.7)	1.03 (0.96-1.11)^
PAI-1 (ng/ml)	32.55 (23.05-49.65)	0.88 (0.77-1.00)^*	35.75 (29.00-50.45)	1.04 (0.86-1.26)^	0.84 (0.68-1.04)^
hsCRP (mg/L) [#]	2.05 (0.79-3.65)	0.85 (0.70-1.03)^	3.48 (1.17-4.32)	0.75 (0.43-1.30)^	1.02 (0.67-1.55)^

Data at baseline presented as mean (SD) or median (p25-p75) and differences as mean (95%CI) or mean ratio (95%CI)^ from logarithmic transformed numbers. * = $p<0.05$, ^= presented as a ratio.
Between-group differences at baseline were determined by un-paired t-test or Mann Whitney U-test, as appropriate.
[#] = hsCRP estimates >10 mg/L were excluded from analysis. N for baseline and difference: liraglutide (n=45, n=42) and placebo (n=20, n=18).
ETP - endogenous thrombin potential; hsCRP - high sensitivity C-reactive protein; PAI-1 - plasminogen activator inhibitor-1; vWF - von Willebrand factor.

Relationship between ETP and anthropometric, metabolic and endocrine parameters

At baseline levels of ETP were positively correlated with BMI ($r=0.40$, $p<0.001$), waist circumference ($r=0.28$, $p<0.05$), diastolic blood pressure ($r=0.27$, $p<0.05$), HOMA2-IR ($r=0.36$, $p<0.01$), triglycerides ($r=0.25$, $p=0.05$), hsCRP ($r=0.41$, $p<0.001$) and PAI1 ($r=0.38$, $p<0.01$), and negatively associated with HDL ($r=-0.24$, $p<0.05$) and SHBG ($r=-0.25$, $p<0.05$). After adjusting for BMI, only PAI-1 ($r=0.50$, $p<0.01$) levels were associated with ETP, an association that was no longer statistically significant when HOMA2-IR, triglycerides and hsCRP were included in the model. There was no association between ETP and androgen levels.

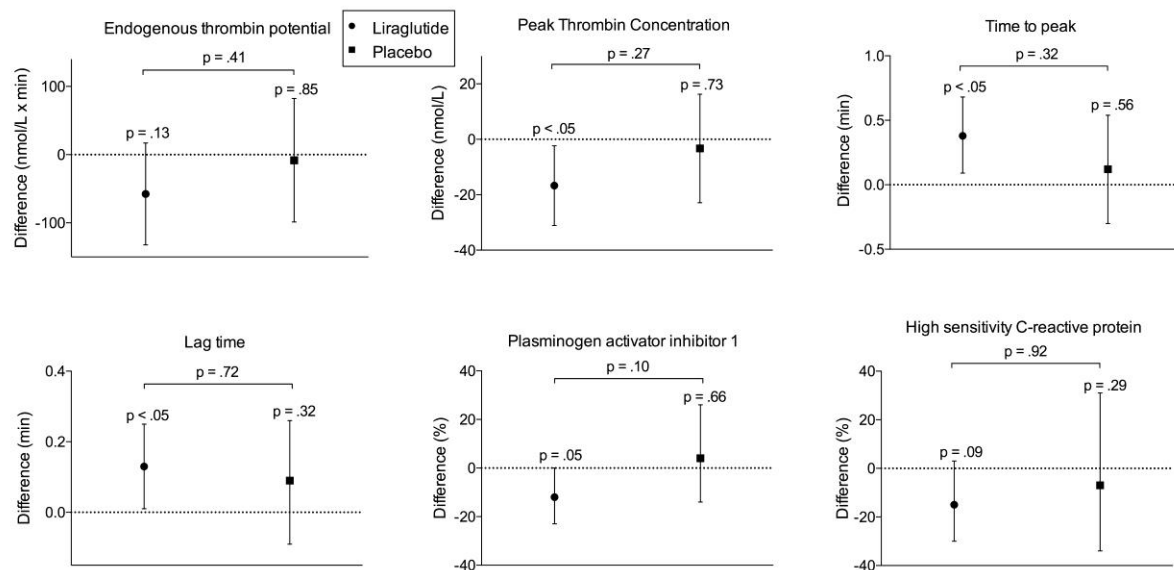


Figure 6. Mean differences in levels of thrombin generation parameters, PAI-1 and hsCRP

Data are presented as mean differences (95%CI). P-values for within-group difference from baseline to 26-week follow-up and for between-group difference in effect size. Bullets represent the liraglutide group and squares represent the placebo group.

Table 4.

Baseline values, within-group and between-group differences for markers of anthropometrics and metabolic markers

	LIRAGLUTIDE		PLACEBO		BETWEEN-GROUP
	Baseline (n=48)	Difference (n=44)	Baseline (n=24)	Difference (n=21)	Crude
Weight (kg)	94.2 (15.4)	-5.2* (-6.6- -3.9)	91.3 (13.6)	0.21 (-1.7-2.1)	-5.2* (-7.5- -3.0)
BMI (kg/m²)	33.3 (5.1)	-1.9* (-2.4- -1.3)	33.3 (4.6)	0.1 (-0.6-0.7)	-1.8* (-2.7- -1.0)
Waist (cm)	103 (11)	-4* (-6- -2)	103 (11)	1 (-2-4)	-6* (-9- -2)
Systolic BP (mmHg)	123 (9)	-2.5 (-5.0-0)	124 (9)	(-2.1 (-5.4-1.3)	-1.6 (-4.8-2.5)
Diastolic BP (mmHg)	79 (8)	-1.4 (-3.5-0.6)	80 (7)	-1.4 (-4.5-1.6)	0 (-2.9-2.8)
HbA1c (mmol/mol)	34.2 (2.8)	-1.3* (-2.0--0.5)	34.6 (3.4)	0.0 (-1.0-1.1)	-1.4* (-2.5--0.3)
Fasting glucose (mmol/L)	5.46 (0.48)	-0.37* (-0.51--0.23)	5.51 (0.54)	-0.15 (-0.34-0.05)	-0.24* (-0.43--0.05)
Fasting insulin (pmol/L)	122.5 (97.5-148.5)	-12.9 (-30.5-4.5)	128.5 (98.5-171.5)	-14.5 (-37.7-8.6)	0.93 [^] (0.75-1.16)
HOMA2-IR	2.29 (1.83-2.84)	-0.27 (-0.58-0.04)	2.42 (1.91-3.20)	-0.28 (-0.70-0.13)	0.93 [^] (0.75-1.14)
LDL cholesterol (mmol/L)	2.83 (0.71)	0.14 (0.04-0.32)	2.99 (0.54)	0.13 (-0.05-0.32)	-0.01(-0.29-0.26)
HDL cholesterol (mmol/L)	1.14 (0.25)	-0.01 (-0.06-0.04)	1.09 (0.28)	0.01 (-0.05-0.07)	-0.01 (-0.09-0.06)
Triglycerides (mmol/L)	1.23 (0.90-1.63)	-0.22 (-0.36--0.09)*	1.15 (0.90-1.47)	-0.11 (-0.37-0.14)	0.94 [^] (0.82-1.07)

Data at baseline presented as mean (SD) or median (p25-p75) and differences as mean (95%CI) or mean ratio (95%CI)[^] from logarithmic transformed numbers. * = p<0.05, [^] = presented as a ratio.

Between-group differences at baseline were determined by un-paired t-test or Mann Whitney U-test, as appropriate.

HbA1c – glycosylated hemoglobin; HDL – high-density lipoprotein; HOMA2-IR – Homeostasis model assessment-estimated insulin resistance ; LDL – low-density lipoprotein

DISCUSSION

Ovarian morphology

In Study I we found 2D TVUS to estimate lower ovarian volume and AFC as compared with 3D TVUS and MRI, whereas there were no differences between 3D TVUS and MRI. Moreover, we found serum AMH to have higher correlation with AFC estimates from 3D TVUS than with estimates from 2D TVUS and MRI.

In accordance with our results, 2D TVUS has been found to measure smaller volumes than 3D TVUS in 214 women with regular menstrual cycles (94). However, the mean difference was minor, -0.16 ml. Inconsistent with our findings, no significant difference was found between volume estimates from 2D- and 3D TVUS in 112 lean PCOS women (95) or in 20 women going through in vitro fertilization (17). The former study used 30° VOCAL, as compared to our, more comprehensive, 15° and the latter study used 3D data for calculation of 2D estimates, which probably influenced the results. Estimates from TVUS have been compared with true volume measured using the Archimedes' principle on oophorectomy specimens. A study on 46 ovaries found 2D-, 3D TVUS estimates and true volume to be 4.0 ml, 5.1 ml and 5.5 ml, respectively (18), which is consistent with a Danish study, finding 27% smaller estimates from 2D TVUS as compared with true volume (19). Presuming MRI to generate the estimates that are closest to the true volume, our findings are consistent with both studies on oophorectomy specimens. Likewise, 2D transvaginal/transabdominal US underestimated ovarian volume as compared with MRI in 39 adolescents with PCOS (96). In contrast, 2D TVUS generated 1.7 ml larger volume estimates than MRI in 99 women with or without PCOS (21). The results might have been influenced by the exclusion of non-ellipsoidal ovaries (21). The main reason for finding 2D TVUS inferior to 3D TVUS and MRI, with regard to volume estimates, is most likely that it assumes the ovary to have a regular, ellipsoidal shape. In contrast, Archimedes' principle, the VOCAL tool of 3D TVUS and Cavalieri's principle on MRI data all use the actual contour of the ovary when estimating the volume. Moreover, finding three planes exactly perpendicular to each other using real-time 2D TVUS can be difficult, whereas using off-line, immobile images as in 3D TVUS and MRI makes identification of the ovarian borders easier and thereby estimates more precise.

With regard to AFC, our results are inconsistent with previous studies. Deb *et al.* found slightly higher AFC with 2D than with 3D TVUS (2.5-3 follicles) in two studies in women planning on undergoing fertility treatment (4,97). In both studies dependent observations (i.e. both ovaries from each woman) were used and one of the studies obtained 2D estimates by scrolling through 3D data (4). Lack of post-processing of 3D data causes underestimation of AFC (4) and is most likely the reason that no difference was found between AFC estimates from 2D- and 3D TVUS in 164 lean women with and without PCOS (95). Different degrees of manual post-processing of 3D TVUS data and inclusion of 1-mm follicles may be the reasons for discrepancies between our results and Leonhardt *et al.*, who found higher AFC with MRI than with 3D TVUS,

mainly due to MRI finding more follicles in the 1-3 mm interval (21). With higher AFC the limits of agreement broaden and the intra/inter-observer ICC decrease, as seen both in 2D- and 3D TVUS in non-PCOS populations (98–100). The risk of double-counting or overlooking follicles is higher when follicles are numerous, small and densely packed. These are all features of the polycystic ovary and most likely the reliability of modalities is lower in polycystic than in normal ovaries, which is also illustrated in the between-modality comparisons in our study.

We found no difference between volume and AFC estimates from 3D TVUS and MRI, suggesting that 3D TVUS gives more accurate estimates than 2D TVUS does. The wider limits of agreement for comparisons of TVUS and MRI than of TVUS modalities indicate that 2D- and 3D TVUS are more consistent than 3D TVUS and MRI. This is probably because the TVUS modalities are more alike than the TVUS and the MRI, the 3D data were obtained directly after 2D estimates were recorded and 2D- and 3D TVUS were evaluated by the same observer.

Ovarian dysfunction

In Study II we observed improved bleeding ratio in both groups at 26-week follow-up, with significantly greater improvement in the liraglutide group. Moreover, we found a trend towards decreased levels of AMH in the liraglutide group, but no between-group difference. Twenty-six weeks of liraglutide treatment caused increased levels of SHBG, and thereby decreased levels of free testosterone. We observed no effect on levels of total testosterone, but in the liraglutide group we found reduced ovarian stroma volume and a trend towards reduced levels of androstenedione, indicating attenuated ovarian androgen production.

Bleeding regularity

The effect of GLP-1 analogs on the menstrual pattern in PCOS is infrequently investigated. Contrasting our results, Jensterle *et al.* found unaltered bleeding ratio in three studies testing liraglutide 1.2 mg/day in PCOS, most likely due to short follow-up (12 weeks) and possibly due to small sample sizes (84–86). Consistent with our results, exenatide, metformin and the combination were all found to improve ovulation rates in a 24-week RCT in oligo-ovulatory PCOS (83). The size of improvement with exenatide was comparable to our findings with liraglutide, despite their population having substantially lower bleeding ratio at baseline. The increased bleeding ratio in our placebo group might be methodological, i.e. bleeding pattern at baseline may be underestimated due to recall bias and the use of a bleeding diary artificially improved the bleeding ratio. Nevertheless, there was a significant difference between the two groups, demonstrating an effect of liraglutide.

Weight loss due to lifestyle intervention or bariatric surgery restores menstrual regularity in overweight women with PCOS (39,101–104). Bariatric surgery immediately restores the defect GLP-1 secretion as seen in patients with DM2 (105), but whether the improved menstrual regularity as seen after bariatric surgery in women with PCOS is due to weight loss, altered glucose metabolism or a direct GLP-1 effect is not clear. Results from a recent animal study indicate a direct effect of GLP-1 on the reproductive axis (106). In healthy rats the GLP-1 receptor was found expressed both in hypothalamus, pituitary and ovaries, with the expression varying through the menstrual cycle. Moreover, GLP-1 treatment amplified the pre-ovulatory LH surge and the number of Graafian follicles (106).

In a small, 12-month study the proportion of women experiencing restored menstruation after bariatric surgery increased with follow-up time (103), indicating the reproductive changes to be mediated by weight loss and improved metabolic state rather than by a direct action of increased GLP-1. Compatible with this Turkmen *et al.* found (albeit non-significant) reduced metabolic disturbances in women with PCOS who responded with ovulation (n=7) to gastric bypass surgery as compared with women still being anovulatory (n=6) six months after gastric bypass (102). Also our findings suggest weight loss, rather than a direct GLP-1 effect to be responsible for the improved bleeding pattern.

In lifestyle intervention studies, women who respond with restored bleeding regularity seem to have greater weight loss and improvement in markers of insulin resistance, than non-responders, indicating insulin to play an important role (38,39,104). Insulin's role is further supported by the fact that metformin therapy cause improved bleeding pattern in normal- and overweight women with PCOS (10). Additionally, in a small lifestyle-intervention study on women with PCOS, reduced nocturnal urinary LH levels were found to be correlated to changes in insulin resistance, rather than changes in BMI (107).

Anti-Müllerian Hormone

The link between weight loss and restored menstrual regularity may be insulin, as it alters androgen levels (28), which are highly correlated to AMH levels (32,34). However, the link between AMH and insulin remains enigmatic. We observed no significant changes in serum AMH, despite improved bleeding pattern, reduced free testosterone and a trend towards decreased stroma volume, which in fact indicates reduced ovarian androgen production. Our finding of unaltered serum AMH levels might be due to high intra-individual variation. Maybe delicate changes in the levels of insulin, testosterone, AMH, etc. are sufficient to alter the intraovarian milieu, restoring menstruation, but too small to be detected in serum samples. Moreover, whether reduced AMH is a cause or a consequence of a restored menstruation is not known and a menstrual cycle improvement might precede a fall in serum AMH. A delayed response is suggested by Fleming *et al.*, who found AMH levels to be unaltered after four months but decreased after eight months of metformin therapy in 82 obese women with PCOS (108).

In PCOS, serum AMH levels have been found to decrease with weight loss and metformin therapy in parallel with improved bleeding pattern (36–38,108). However, conflicting data exist (39,109), where some divergences might be explained by differences in baseline AMH.

With weight loss, the reduced intra-ovarian androgen production, which is related to the decreased hyperinsulinemia, might reduce the number of AMH-secreting pre-antral and early antral follicles, thereby decreasing intraovarian AMH levels and allowing some follicles to go through ovulation. Another possibility could be a direct effect where attenuated androgen levels cause reduced AMH production by the granulosa cells. The relationship between AMH and androgens is well described from cross-sectional studies (31–33). Moreover, Nybacka *et al.* found reduced levels of free testosterone, rather than reduced BMI, to be the strongest factor predicting decreased serum AMH (36). This was in accordance with our results.

Androgen excess

We found liraglutide to cause increased SHBG and decreased free testosterone, but unaltered total testosterone levels. In accordance with this, SHBG levels increased, free testosterone levels decreased and total testosterone levels were unaltered with 12 weeks of liraglutide in an open-labelled RCT comparing liraglutide with the combination of liraglutide and metformin in obese women with PCOS (89). In the (liraglutide + metformin)-group both total and free testosterone levels decreased and SHBG levels increased (89). Inconsistent with our results, 24-weeks of exenatide were found to reduce levels of total testosterone, in a population with markedly higher baseline testosterone levels than our population's (83). Short-term (12 weeks), open-labelled studies, testing 1.2 mg/day liraglutide in overweight women with PCOS, found no effect on either total testosterone or SHBG levels (84,86,87).

The rise in SHBG levels observed in our study is most likely due to reduced visceral adipose tissue, which also was observed in a lifestyle intervention study on 107 overweight premenopausal women (30). Also, improved glucose metabolism, might have had an impact on the SHBG levels in our study; Despite unaltered insulin levels, we observed decreased levels of HbA1c, suggesting an effect of liraglutide on the insulin levels. Thus, we found a marked effect on levels of SHBG, but only a trend towards attenuated ovarian androgen production. Additionally, we found a substantial weight loss and reduced waist circumference, but no significant effect on fasting insulin. This is opposite to what was found in a meta-analysis on metformin intervention in PCOS, where BMI and SHBG levels were unaltered, whereas both fasting-insulin and total testosterone levels decreased (10). Discrepancies might be due to different pharmacodynamics.

Theoretically the combined treatment of GLP-1 analog and metformin, making use of the additive, or maybe synergistic, effect would be superior with regard to altering BMI, metabolic and endocrine disturbances, which also has been reported in a recent study (89).

Attenuated levels of total testosterone and increased levels of SHBG has also been found in studies on lifestyle intervention and bariatric surgery in overweight women with PCOS (39,102,103), most likely mediated via reduced adipose tissue mass and insulin resistance.

As earlier pointed out, we found a trend towards decreased total and stroma volume in the liraglutide group, compared with placebo. In agreement with this, a single-armed study found a 5% diet-induced weight loss to reduce ovarian volume in overweight women with anovulatory PCOS (101). In contrast, no statistically significant change was seen in ovarian volume after gastric bypass surgery despite a 25% weight loss and reduced levels of total testosterone (102), probably due to a small population (n=13). Metformin intervention has been found to reduce ovarian volume (110) and stroma/total area ratio in PCOS (111), but conflicting results exist (108).

In the quoted studies 2D TVUS was used, only two used blinded observers (101,111) and only one addressed the inter- and intra-observer variation (101). Moreover, the issue of whether the reduced ovarian volume is due to decreased follicular or stromal volume was only addressed in one study (111).

It is most likely the improved metabolic status, with decreased insulin levels, that attenuates the ovarian androgen production, thereby reducing the stromal volume. In PCOS, the stroma volume is associated with excess theca cell steroid production, as measured by androstenedione and testosterone levels (112), a relationship we can confirm (data not shown).

Thrombin generation, endothelial dysfunction and low-grade inflammation

We found no effect of 26 weeks of liraglutide treatment on ETP in either group, but decreased peak thrombin concentration as well as increased lag time and time to peak in the liraglutide group at 26-week follow-up. However, we observed no significant between-group differences in any thrombin generation parameter. At 26-week follow-up PAI-1 levels decreased in the liraglutide group, but there was only a trend towards decreased PAI-1 levels in the liraglutide group as compared with placebo.

To our knowledge, the effect of GLP-1 analog intervention on in vivo thrombin generation has never been explored in humans before. Kahal *et al.* investigated the effect of 1.8 mg/day liraglutide on platelet function in 24 overweight women, 13 with and 11 without PCOS, in a single-armed six-month trial (88). Reduced BMI and HOMA-IR were observed in both groups, as were increased lag phase and reduced “clot lysis area”, a compound measure of clot formation, density and lysis. This is accordant with findings from a recent in vitro and animal study on the antithrombotic effect of the GLP-1 analog exenatide (113), where incubation with exenatide reduced thrombin-induced platelet aggregation and thrombus formation in both human and murine whole blood. Moreover, a single i.v. bolus of exenatide inhibited in vivo thrombus formation in normo- and hyperglycemic mice, indicating a direct effect of the GLP-1 analog (113). Our TGT was performed

in platelet-poor plasma and therefore the possible direct effect of liraglutide on platelet function was not assessed and so our results are not straightforwardly comparable with results from Kahal *et al.* In a randomized trial investigating 12 months of metformin, COC or a combination in 90 women with PCOS, Glintborg *et al.* did not find metformin to affect TGT parameters, despite a small weight loss (66,78). Thrombin generation was significantly increased in the COC group and to a lesser extent in the COC + metformin group, suggesting a protective effect of metformin.

Our hypothesis that a weight loss and/or improved glucose metabolism would cause decreased thrombin generation measured by TGT, has been verified in other populations (93,114,115). Thrombin generation was normalized two years after bariatric surgery in 36 morbidly obese participants (29 females) (114). Mean weight loss was 41 kg and reduced HOMA-IR, HbA1c, triglycerides and hsCRP were observed. In 27 overweight children, one year of lifestyle intervention reduced BMI by 5% and normalized the TGT parameters (93) and in moderately overweight men, with age 20-40 years, three months of daily exercise lowered BMI by 3-4% and ETP by 170-185 nmol/min, as compared with controls (n=53) (115). Reasons for inconsistency, regarding ETP, between our results and these studies could be the extreme weight loss in the first (114), the lack of control group in the second (93) and the different intervention in the third study (115). Our population might have been too healthy, with regard to CVD risk, to detect an effect on thrombin generation, despite mean BMI 33 kg/m² at baseline. Nevertheless, the fact that some of the TGT parameters were altered in the liraglutide group indicates that there might be an effect of the treatment, but the population might be too small to detect it; In this respect, the SD of ETP used for sample size calculation was lower than the actual SD measured, indicating the possibility of a type 2 error.

At baseline, we found thrombin generation, measured as ETP, to be associated with waist circumference, HOMA2-IR, levels of hsCRP, PAI-1 and SHBG, but after adjusting for BMI the associations disappeared and only levels of PAI-1 were correlated with ETP. Similarly, in a previous study on women with PCOS our group found higher ETP in an overweight and insulin resistant subgroup (69). Also here the difference disappeared after adjustment for waist circumference, hsCRP and triglycerides, indicating the thrombin generation to be mediated via obesity-related low-grade inflammation. The relation between ETP and abdominal obesity as well as hsCRP is also observed in non PCOS-populations (71–73). Glintborg *et al.* found higher thrombin generation, measured as ETP, in women with PCOS as compared to age- and BMI matched controls, also after adjusting for trunk fat mass and the acute phase protein fibrinogen, indicating that other factors than abdominal obesity have influence on thrombin generation in women with PCOS (66). We did not find ETP to be independently associated with other covariates than BMI and PAI-1, especially not with androgens or SHBG.

In our study liraglutide reduced the body weight and metabolic disturbances sufficiently to improve bleeding pattern, but not to reduce levels of hsCRP. The reason for not finding an effect on levels of hsCRP and only a trend towards decreased levels of PAI-1, despite a 5% weight loss, reduced waist circumference and levels of HbA1c might be that our participants were too metabolically healthy at baseline. In 13 women with PCOS Kahal *et al.* found 1.8 mg/day liraglutide for six months to reduce markers of endothelial dysfunction, including levels of hsCRP (88). Dissimilarities could be explained by higher age (mean 34 years), higher BMI (mean 38 kg/m²), higher prevalence of the metabolic syndrome (53%) and higher mean baseline-hsCRP among their participants. Also in an obese population (n=2590), major reductions in levels of hsCRP and PAI-1 were found with liraglutide (3.0 mg/day) as compared with placebo in a 56-week RCT (13). The trend towards reduced levels of PAI-1 with liraglutide could be due to reduced body weight and/or reduced plasma glucose or due to a direct effect on the endothelium. In vitro liraglutide has been found to attenuate hyperglycemia- and TNF α -induced expression of PAI-1 as well as endothelial adhesion molecules in human endothelial cells (116,117).

Overall evidence suggests that reducing the body weight and the metabolic dysfunction through lifestyle intervention and/or metformin therapy lessens the endothelial dysfunction and low-grade inflammation in PCOS. In studies without non-interventional control groups, diet- and/or exercise related weight loss of 8-11% reduced levels of PAI-1 (118), whereas weight loss <5% did affect other markers of endothelial dysfunction, but not levels of hsCRP and PAI-1 in obese PCOS (119,120). In a double-blind RCT on exercise, COC and placebo (vitamin pills) in 139 women with PCOS, exercise attenuated endothelial dysfunction measured as intima media thickness and levels of hsCRP and PAI-1 (121). However, when compared with placebo only the effect on intima media thickness remained significant (121). Also single-armed (122) and open-labelled (123) studies, comparing metformin with COC in women with PCOS found metformin to reduce levels of hsCRP and PAI-1. In contrast, in a comprehensive placebo-controlled crossover trial on 25 women with PCOS and mean BMI 35kg/m², 12 weeks of metformin did not affect levels of hsCRP and PAI-1 but other markers of endothelial dysfunction (124). In general, different diagnostic criteria, baseline BMI and degree of abdominal obesity make results difficult to compare.

Strengths and limitations

Study I - Ovarian morphology

The study has several strengths: The latest technology was used, all three scans were performed within four hours and overnight fasting minimized MRI artifacts due to bowel movements. Nevertheless, it also had some flaws. Visceral adipose tissue is associated with poor image quality and since the mean BMI for

participants in this study was high (32.7 kg/m²) the quality of scans were probably suboptimal. This reflects a true problem in the clinical setting and proves our results applicable in the standard PCOS population. A junior doctor performed the TVUS and the effect of a “learning curve” cannot be neglected. Nonetheless, the observer had three months intensive TVUS training, including ultrasound courses, prior to the study and a previous report found no significant learning curve for the VOCAL modality (125). The observer who assessed the 3D data was not strictly blinded to 2D TVUS estimates, as 3D data and 2D estimates were obtained at the same session. This might have caused bias, resulting in less variation between 2D- and 3D TVUS. However, this effect was minimized as the assessment of 3D data was done a minimum of two weeks after the 2D TVUS. Another limitation was that intra- and inter-observer agreement was only assessed for 3D TVUS and MRI and not for 2D TVUS. However, this would require three consecutive TVUS, which we did not find patient friendly.

Study II and Study III - The LIPT study

The LIPT study has several strengths: The placebo-controlled RCT design, a low dropout rate and a recruitment strategy securing external validity. Moreover, we used the gold standard double mass spectrometry for estimation of serum androgens and all blood sampling were performed in the morning after overnight fasting. Nevertheless, the study has some limitations. One weakness of Study II was that participants did not keep a bleeding diary prior to study start and therefore recall bias might have influenced bleeding ratio at baseline. Bleedings rather than ovulations were measured and therefore the impact on menstruation might be overestimated in both groups. Additionally, the bleeding pattern might have been influenced by the copper IUD that, for safety reasons, was mandatory for participants.

The main limitation of Study III was the sample size. The variance of the primary outcome (ETP) was higher than expected, implicating that the study actually was somewhat underpowered for this parameter. Moreover, our population may have been too healthy. To examine the effect of liraglutide in an “every day population” we may have ended up including relatively “metabolically healthy” obese women, which may have influenced the results regarding thrombin generation, endothelial dysfunction and low-grade inflammation. Including women at higher risk, i.e. of higher age or women diagnosed using the *National Institute of Health* criteria or the revised AE/PCOS Society criteria instead of the Rotterdam criteria, may decrease external validity, but might have resolved this issue (47). Moreover, many women with PCOS experience weight cycling and including women who were not stable in weight in the six months just prior to the study might have blurred the results. However, this flaw was obviously present in both groups. Furthermore, as most of the participants were oligo-/amenorrheic, examinations were not performed in early follicular phase as appropriate. This probably biased the results regarding gonadotropins and female sex steroids and might also have influenced the results on ovarian morphology and thrombin generation (126).

CONCLUSION

In conclusion, we found 3D transvaginal ultrasound (TVUS) to estimate higher ovarian volume and antral follicle count (AFC) as compared with 2D TVUS, whereas there was no difference between 3D TVUS and MRI. Additionally, AFC from 3D TVUS had the highest correlation with serum AMH, compared with 2D TVUS and MRI. Taken together, this suggests 3D TVUS to be a modality appropriate for future use in PCOS research.

We demonstrated that 26 weeks of liraglutide treatment altered ovarian function indicated by improved menstrual regularity. Non-significant reductions in ovarian stroma volume and levels of androstenedione suggest decreased ovarian androgen production. This, in combination with increased SHBG, caused reduced levels of free testosterone.

Moreover, we found liraglutide intervention to cause minor alterations in thrombin generation test parameters, but not to affect overall thrombin generation in overweight women with PCOS. Also a trend towards beneficial effects on the fibrinolytic capacity was demonstrated. The findings of unaltered VTE and CVD risk markers might be due to a too small and relatively, metabolically healthy population.

Taken together, our results suggest that liraglutide has the potential for being an appropriate treatment option in an overweight PCOS population; however, larger, placebo-controlled trials are needed to further establish the role of GLP-1 analogs in treating PCOS.

PERSPECTIVES

3D transvaginal ultrasound

Given that PCOS is a disorder with several serious comorbidities (e.g. infertility, DM2, CVD and endometrial malignancy) an incorrect diagnosis could cause a great deal of unnecessary anxiety and an accurate and certain diagnosis is of great importance. The use of modern 2D ultrasound scanners require revised thresholds for AFC for the Rotterdam criteria, where both 19 and 25 follicles have been proposed (14,15). However, with increasing AFC, reliability decreases. Here 3D TVUS appears useful, as it has higher reliability than 2D TVUS (4,99). If 3D TVUS is to be used for PCOS diagnostics, specific thresholds for ovarian volume and AFC are needed. However, at the moment, being rather expensive and time-consuming, 3D TVUS' role is first and foremost in the research setting, where it has several advantages:

- Calculation of stroma volume as a qualitative measure of ovarian androgen production.
- Storage of 3D datasets, which can secure data completeness and be used for “blinding” in open-labelled trials, i.e. data can be obtained at one time and assessed at another.
- High reliability, which is especially important in PCOS where the extraordinary high AFC can cause inaccurate estimates.

We found estimates from 3D TVUS and MRI to be comparable and serum AMH to have slightly higher correlation with AFC from 3D TVUS than from MRI. If this could be replicated in future studies, 3D TVUS, being more patient friendly and less expensive than MRI, might be used as a new gold standard for ovarian morphology in research settings.

Liraglutide

Given that obesity is increasing worldwide and that the ovarian and metabolic components of PCOS are disorientated by obesity, there is an obvious need for adjuvant pharmacological treatments to be used in combination with lifestyle intervention, aiming at weight reduction in this population. Liraglutide could fill this gap, as a proper treatment in overweight and obese women with PCOS, without pregnancy wish. We found the drug to be well tolerated and to alter both the ovarian and metabolic dysfunction.

In PCOS, amelioration of the reproductive function with restored bleeding regularity and decreased levels of free testosterone is often achieved with the use of COC. However, this intervention is truly controversial as both COC and PCOS are linked to VTE (6). This unfortunate side effect, probably dependent on the progestin content of the COC, is also seen as increased thrombin generation in COC-users (61,66). We found liraglutide to modify the ovarian dysfunction without increasing the thrombin generation, suggesting liraglutide together with a progestin-only-contraceptive pill or levonorgestrel IUD to be a more appropriate and safe treatment in the obese woman with PCOS.

A clear advantage of GLP-1 analogs is the appetite modifying effect, which may alter the disturbed appetite regulation in women with PCOS that previously has been described by Hirschberg *et al.* (127). Accordingly, eating behavior was improved in a single-armed study on liraglutide in obese women with PCOS, where self-reported scores of uncontrolled eating and emotional eating were significantly reduced (87).

Published trials on liraglutide in PCOS use doses of 1.2-1.8 mg/day. Nonetheless, the weight loss potential of liraglutide is dose-dependent (11) and since the onset of our study the use of liraglutide 3.0 mg/day has been approved for weight reduction in obesity (BMI >30 kg/m²) in the USA and several European countries. Additionally, previous trials have observed greater weight loss and metabolic amelioration using the combination of a GLP-1 analog and metformin as compared with single-drug intervention (83,89). Here the two drugs' different pharmacodynamics may act additive or maybe even synergistic. The combination of a GLP-1 analog and metformin, together with lifestyle intervention, including hypo-caloric diet and exercise could serve as a treatment in overweight women with PCOS. One possible approach might be to initiate a substantial weight loss with a combination of lifestyle intervention, metformin and liraglutide (3.0 mg/day) for 6-12 months and thereafter to maintain the weight with lifestyle intervention and liraglutide (0.6 mg/day).

Despite encouraging findings in this thesis and in the studies published since we undertook the LIPT study, it is clear that GLP-1 analog intervention has some limitations. The GLP-1 analog based therapies have been used for a decade in patients with DM2, but intervention in younger populations is a rather new practice and long-term effects are unknown. As with all novel interventions long-term effects, also the rare ones, should be evaluated. With regard to serious adverse events, evidence from studies with numerous, accumulated patient-years indicate no increased risk of pancreatitis (128) or pancreatic malignancies (129) with GLP-1 based therapies, a concern that previously has been raised. However, for obvious reasons there are no data on patients receiving GLP-1 analogs from young age and over several decades.

Moreover, GLP-1 analogs are contraindicated in pregnant women; thus, pre-pregnancy counselling is a must when using GLP-1 analogs in this population. Possibly, GLP-1 analog intervention can be used pre-pregnancy in overweight women with PCOS, to achieve a weight loss and thereby increase the chance of conceiving. Despite having a possible direct effect on the reproductive axis, as demonstrated in rats (106), liraglutide intervention may be restricted to overweight and obese women with PCOS, as lean women might experience an unwanted anorectic effect.

It is well-known that obese women are at risk of gallbladder stones and it should be addressed that initiating a rapid weight loss can provoke gallbladder stone attacks, which in our study was seen both in the liraglutide and placebo group. Additionally, in our study it was evident that liraglutide facilitated a great weight loss in some women and a more modest weight loss in others; a phenomenon that is well known from the clinical setting and also has been described by others (130). Jensterle *et al.* suggested GLP-1 receptor polymorphism

to be one of the reasons for the inter-individual differences in the weight lowering potential of liraglutide (130), i.e. maybe liraglutide intervention is not for all.

Future research

The studies in this thesis have identified several areas of interest:

- A large cross-sectional study including an unselected population is needed to establish polycystic ovarian morphology thresholds (volume and AFC) for 3D TVUS.
- For up-to-date 2D TVUS as well as for 3D TVUS, intra- and inter-observer ICC should be established in a large PCOS population, encompassing women with a wide range of BMI and AFC.
- Larger, long-term placebo-controlled RCTs are warranted to further investigate the role of liraglutide in treating PCOS. Not only the metabolic, but also the ovarian dysfunction should be addressed.

The following should be investigated:

- The combined intervention of intensive lifestyle intervention, metformin and liraglutide.
- Metformin and liraglutide should be compared in a double-blind RCT using minimum 1.8 mg/day liraglutide and with minimum six months follow-up.
- A liraglutide regime including an initial dose of 3.0 mg/day (after up-titration) and a subsequent maintenance dose of 0.6 mg/day.
- The effects on body composition, metabolic and reproductive parameters of liraglutide compared to other anti-obesity medications, e.g. orlistat, could be of interest.
- Additionally, comparing the effect of bariatric surgery with the effect of combined lifestyle intervention and liraglutide 3.0 mg/day in morbidly obese women with PCOS might be interesting. Bariatric surgery effectively reduces body weight and ameliorates metabolic dysfunction, but can have unpleasant metabolic, endocrine and obstetric complications (131). GLP-1 analogs may be used, postponing bariatric surgery in obese women with pregnancy wish.
- Finally, the use of long acting GLP-1 analogs allowing for administration once weekly could improve the compliance and thus the outcome. In this context it is interesting that once-weekly semaglutide in phase 2 studies seems to induce a greater weight loss than liraglutide (132).

SUMMARY

Ten percent of premenopausal women have polycystic ovary syndrome (PCOS), a complex endocrine disorder encompassing both an ovarian and a metabolic dysfunction. Assessment of the ovarian morphology is pivotal for the PCOS diagnosis; here 3D transvaginal ultrasound (TVUS) has several advantages, but the modality has never been validated against the traditional 2D TVUS in a PCOS population. The diagnostic Rotterdam criteria: oligo-/anovulation, hyperandrogenism and polycystic ovarian morphology, address the ovarian component, whereas the metabolic dysfunction covers insulin resistance and obesity, which are associated with low-grade inflammation, endothelial dysfunction and increased risk of venous thromboembolism (VTE) and cardiovascular disease (CVD) in later life. Glucagon-like peptide-1 (GLP-1) analog intervention causes weight loss and reduces markers of CVD risk in patients with type 2 diabetes and obesity. Currently, the role of GLP-1 analogs in treating PCOS is being mapped out.

One objective of this PhD thesis was to validate 3D TVUS against 2D TVUS and magnetic resonance imaging (MRI), with regard to ovarian morphology, in a PCOS population (Study I). In addition, we investigated the effect of intervention with the GLP-1 analog liraglutide on ovarian function (Study II) as well as on markers of VTE and CVD risk: Thrombin generation, endothelial dysfunction and low-grade inflammation (Study III).

Study I was cross-sectional, using baseline data from Study II, with 2D TVUS, 3D TVUS, MRI performed and serum anti-Müllerian hormone (AMH) determined in 66 women with PCOS. 3D TVUS estimated higher ovarian volume and antral follicle count (AFC) as compared with 2D TVUS, but was equivalent to MRI. Moreover, serum AMH had the highest correlation with AFC estimates from 3D TVUS, followed by MRI and 2D TVUS. Study II + III were generated through a double-blind RCT where 72 women with PCOS were randomized to 26 weeks of treatment with 1.8 mg/day liraglutide or placebo. Study II demonstrated liraglutide intervention to improve menstrual regularity and to reduce free testosterone via increased SHBG levels and possibly by altered ovarian androgen production. In Study III we found 26 weeks of liraglutide intervention not to alter over-all thrombin generation or low-grade inflammation despite a 5% weight loss. There was a trend towards improved fibrinolysis as measured by plasminogen activator inhibitor-1.

In conclusion, 3D TVUS appears superior to 2D TVUS and could be advantageous in research settings. If used for PCOS diagnostics, new diagnostic thresholds are needed for ovarian volume and AFC. Moreover, liraglutide might be a proper treatment modality for overweight and obese women with PCOS, ameliorating ovarian dysfunction and reducing body weight. However, liraglutide did not significantly alter VTE and CVD risk markers and future research should clarify whether other doses or regimes could do so.

RESUMÉ

Ti procent af premenopausale kvinder har polycystisk ovarie syndrom (PCOS); en kompleks, endokrin lidelse som omfatter både en ovariel og en metabolisk dysfunktion. Vurdering af ovariemorfologi er central i PCOS diagnostikken. Her har 3D transvaginal ultralyd (TVUL) flere fordele, men modaliteten er aldrig blevet valideret overfor den traditionelt brugte 2D TVUL, i en PCOS population. De diagnostiske Rotterdam-kriterier: oligo-/anovulation, hyperandrogenisme og polycystisk ovariemorfologi, adresserer den ovarielle komponent, mens den metaboliske dysfunktion dækker over insulinresistens og fedme, som er associeret til *low-grade inflammation*, endotelial dysfunktion og øget risiko for venøs tromboembolisme (VTE) og på sigt for kardiovaskulær sygdom. Intervention med glucagon-like peptide-1 (GLP-1) analoger medfører vægttab og reduktion i kardiovaskulære risikomarkører hos patienter med type 2 diabetes og fedme. Aktuelt kortlægges GLP-1 analogers rolle indenfor behandlingen af PCOS.

Et af formålene med denne ph.d.-afhandling var at validere 3D TVUL overfor 2D TVUL og magnetisk resonans scanning (MR), mht. ovariemorfologi i en PCOS population (Studie I). Desuden undersøgte vi effekten af intervention med GLP-1 analogen liraglutide på ovariefunktion (Studie II), samt på risikomarkører for VTE og kardiovaskulær sygdom: trombin generation, endotelial dysfunktion og *low-grade inflammation* (Studie III), hos kvinder med PCOS.

I Studie I, som brugte baseline data fra Studie II, fik 66 kvinder med PCOS foretaget 2D TVUL, 3D TVUL og MR, samt målt serum anti-Müllerisk hormon (AMH). 3D TVUL estimerede større ovarievolumen og højere *antral follicle count* (AFC) sammenholdt med 2D TVUL, men var sammenlignelig med MR. Derudover korrelerede serum AMH højest med AFC estimeret fra 3D TVUL, efterfulgt af estimeret fra MR og 2D TVUL. Studie II + III udgjorde et dobbelt-blindet RCT hvor 72 kvinder med PCOS blev randomiseret til 26 ugers intervention med liraglutide eller placebo (1.8 mg/dag). I Studie II fandt vi at liraglutide behandling medførte forbedret blødningsmønster og reduceret niveau af frit testosteron. Det sidste via øget niveau af SHBG og muligvis via nedsat ovariel androgenproduktion. I Studie III fandt vi at 26 ugers liraglutide behandling ikke påvirkede trombin generation eller *low-grade inflammation*, på trods af et vægttab på 5%. Der var en trend mod forbedret fibrinolyse, målt som plasminogen activator inhibitor-1.

3D TVUL fremstår 2D TVUL overlegen og kan være fordelagtig i forskningssammenhænge. Hvis brugt til PCOS diagnostik, så er der behov for nye cut-of værdier for ovarievolumen og AFC. Liraglutide kan være en behandlingsmulighed for overvægtige og fede kvinder med PCOS, hvor den bedrer ovariefunktion og reducerer kropsvægt. Behandling med liraglutide påvirkede ikke VTE og CVD risikomarkører signifikant og fremtidig forskning må afklare om andre doser eller behandlingsregimer kan gøre dette.

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Ovarian morphology in polycystic ovary syndrome: estimates from 2D and 3D ultrasound and magnetic resonance imaging and their correlation to anti-Müllerian hormone

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Abstract

Background: Due to improved ultrasound scanners, new three-dimensional (3D) modalities, and novel Anti-Müllerian hormone (AMH)-assays, the ultrasound criteria for polycystic ovarian morphology are under debate and the appropriate thresholds are often requested.

Purpose: To quantify the differences in estimates of ovarian volume and antral follicle count (AFC) from two-dimensional (2D) and 3D transvaginal ultrasound (TVUS) and magnetic resonance imaging (MRI).

Material and Methods: A cross-sectional study on 66 overweight women with polycystic ovary syndrome (PCOS) according to Rotterdam criteria. Ovarian volume and AFC were estimated from MRI, 2D TVUS, and 3D TVUS, and serum AMH levels were assessed. Bland–Altman statistics were used for comparison.

Results: Participants had a median age of 29 years (age range, 19–44 years) with a mean BMI of 32.7 kg/m² (SD 4.5). Ovarian volume from 2D TVUS was 1.48 mL (95% confidence interval [CI], 0.94–2.03; $P < 0.001$) and 1.25 mL (95% CI, 0.62–1.87; $P < 0.001$) smaller than from 3D TVUS and MRI, respectively. AFC from 2D TVUS was 18% (95% CI, 13–23; $P < 0.005$) and 16% (95% CI, 6–25; $P < 0.005$) smaller than estimates from 3D TVUS and MRI, respectively. Correlations between AMH and AFC from 2D TVUS, 3D TVUS, and MRI were 0.67, 0.78, and 0.70, respectively ($P < 0.001$ for all).

Conclusion: In an overweight PCOS population, 2D TVUS underestimated ovarian volume and AFC as compared with 3D TVUS and MRI. Serum AMH correlated best with AFC from 3D TVUS, followed by MRI and 2D TVUS. The advantage of 3D TVUS might be of minor clinical importance when diagnosing PCOS, but useful when the actual AFC are of interest, e.g. in fertility counseling and research.

Keywords

3D transvaginal ultrasound, magnetic resonance imaging (MRI), polycystic ovary syndrome (PCOS), polycystic ovaries, Anti-Müllerian hormone

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Introduction

Polycystic ovary syndrome (PCOS) is a common disorder among women of reproductive age, with prevalence of 5–10% (1,2). The syndrome is an exclusion diagnosis defined by minimum of two out of the three Rotterdam criteria: oligo-/anovulation,

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hyperandrogenism, and polycystic ovaries (PCO) on transvaginal ultrasound (TVUS) (1). Since the criteria were established in 2003 there has been a significant development of ultrasound scanners, and the diagnostic PCO criteria are under discussion. This is an ongoing debate with several proposals of higher thresholds for the antral follicle count (AFC) (2–4) as well as suggestions of using Anti-Müllerian hormone (AMH) as a biochemical marker of PCO (2). AMH is secreted by the granulosa cells of early developing follicles and serum levels correlate highly with AFC (2).

As PCOS is associated with infertility, psychiatric disorders, and metabolic co-morbidities (5), an accurate and certain diagnosis is important. Due to this, and since ovarian morphology remains pivotal for the PCOS diagnosis, we are in need of a reliable, precise imaging method as well as new thresholds for ovarian volume and AFC when using up-to-date two-dimensional (2D) scanners and newer three-dimensional (3D) modalities. Moreover, validation of 2D and 3D TVUS with a gold standard seems appropriate. Previously two studies have evaluated TVUS ovarian volume estimates with the gold standard of Archimedes' principle after oophorectomy (6,7). Oophorectomy is uncommon in young women and studies comparing TVUS with ex-vivo specimens are rare. Magnetic resonance imaging (MRI) has an advantage when examining adolescents and obese women, has high resolution and no radiation risk and might serve as a gold standard. Several studies on ovarian morphology in 3D TVUS (4,8,9) and MRI (10,11) are published, only one comparing the modalities in adults (12). 2D and 3D TVUS are readily compared with respect to ovarian morphology, predominantly in non-PCOS populations (13–16).

The question of which thresholds to be used with improved imaging modalities is often raised. Therefore, we aimed to compare estimates for ovarian volume and AFC from 2D and 3D TVUS with MRI as a gold standard and to quantify differences between the modalities. Furthermore, we investigated the correlation between serum AMH and AFC from the three modalities.

Material and Methods

Design

This is a cross-sectional study using baseline data from a randomized clinical trial (clinicaltrials.gov: nct02073929) at Herlev Gentofte Hospital, University of Copenhagen, Denmark (17). Data were collected from March 2014 to December 2015. The trial was approved by the Danish Data Protection Agency and the regional Ethics Committee of the Capital Region of

Denmark (ID: H2-2013-142, EudraCT: 2013-003862-15) and performed in accordance with General Clinical Practice guidelines and the declaration of Helsinki. All subjects gave oral and written consent prior to inclusion.

Population

Sixty-six women with PCOS according to the Rotterdam criteria (1) were recruited from social media, outpatient clinics and private practicing gynecologists. Inclusion criteria are published elsewhere (17). In brief they were age ≥ 18 years, premenopausal, body mass index (BMI) $\geq 25 \text{ kg/m}^2$, and/or insulin resistance (fasting proinsulin c-peptide $> 600 \text{ pmol/mL}$). Exclusion criteria were pregnancy or breastfeeding, smoking > 10 cigarettes/day, diabetes, hypertension, cancer, and inflammatory disease. The use of hormonal contraceptives within six weeks prior to assessment and/or antidiabetic or antiandrogenic medications within three months prior to assessment led to exclusion. Prior to inclusion, participants had a medical interview, a physical exam, including 2D TVUS, and blood tests to confirm the PCOS diagnosis and to rule out other etiologies to irregular bleeding and hyperandrogenism. PCOS was defined as ≥ 12 follicles (2–9 mm) and/or volume $> 10 \text{ mL}$, in at least one ovary assessed by 2D TVUS (1). TVUS and MRI were performed on the same day, within 4 h, after an overnight fast. Follicles of 2–9 mm were included in the AFC. One woman had a 3.2 mL ovarian cyst, which was subtracted from the total volume in TVUS and MRI estimates.

2D and 3D transvaginal ultrasound

TVUS were performed by the same observer (MN) using a Voluson E6 scanner, with a 5–9 MHz vaginal transducer (GE Healthcare, Chicago, IL, USA). The investigator was blinded to the MRI results. For 2D volume estimates, measurements of the maximal longitudinal, transverse, and anteroposterior diameter were obtained from two images, perpendicular to each other. Volume was calculated using the formula for an ellipsoid: $v = \text{length} \times \text{width} \times \text{height} \times 0.523$. AFC was estimated in two sweeping motions from margin to margin in two planes perpendicular to each other. The mean of the two AFC was recorded.

3D data were obtained with the 3D facility, where an automatic swipe (angle 90°) through the ovary collects multiple 2D images for construction of a 3D set. The 3D data were post-processed offline using the software program 4D View (GE Healthcare). For ovarian volume the “Virtual Organ Computer-aided Analysis” tool (VOCAL) was used, where outlining the contour of the ovary in 12 image sections rotating in 15° steps around the y-axis creates a 3D model. The software

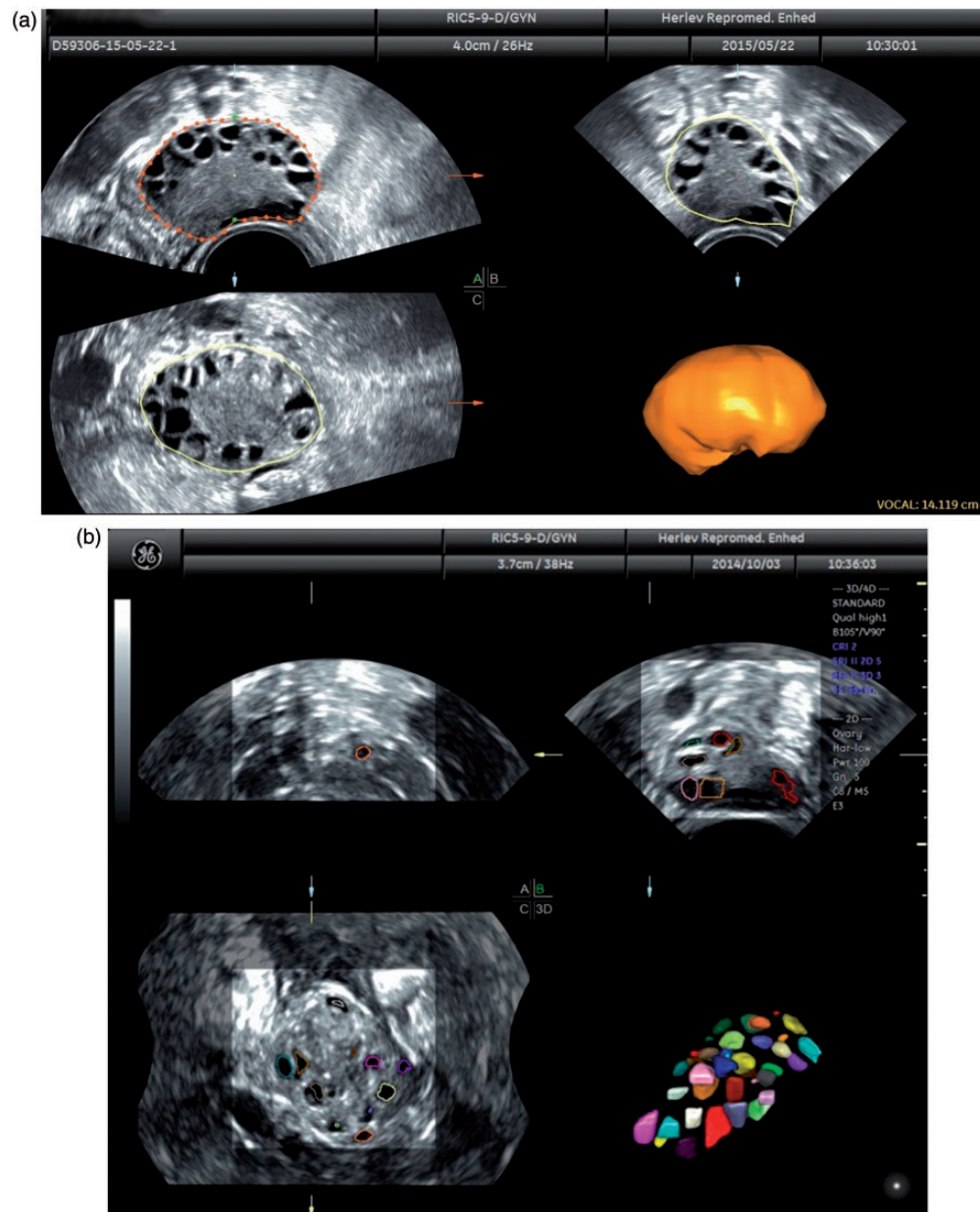


Fig. 1. 3D transvaginal ultrasound. (a) The VOCAL tool, used for estimation of ovarian volume; (b) the SonoAVC tool, used for estimation of antral follicle count.

calculates the volume inside the marked contour (Fig. 1). The AFC from 3D data was obtained with the “Sonography-based Automated Volume Count” tool (SonoAVC). SonoAVC detects and color-codes hypo-echogenic areas (follicles) in the dataset and provides estimates of dimensions and volume for each of them (Fig. 1). Images can be post-processed manually, e.g. if follicles are missed out or if multiple follicles are perceived as one by the SonoAVC tool. Hence, AFC estimation with SonoAVC was “semi-automatic.” 3D data were analyzed blinded to the 2D TVUS results, at least two weeks after the TVUS.

MRI

MRI scans were performed using an Achieva 3.0T MR Imaging System (Philips Medical systems, Best, The Netherlands) and a sense cardiac coil. T2-weighted axial slices (2 mm) in the transverse plane of the lower abdomen and pelvis obtained images for estimation of ovarian volume and AFC. Images were analyzed on a Philips ViewForum workstation (Philips Medical systems) using “Segmentation tool” in “Volume analysis.” A single observer (AB), who was blinded to the TVUS results, analyzed all images. The ovarian contour was outlined

in 2 mm thick sections and volume was automatically calculated from the areas of the sections. For AFC estimates follicles were counted, scrolling through the 2 mm sections.

Anti-Müllerian hormone

Blood samples were drawn between 08:00 and 10:00 on the day of scanning. After overnight fasting and 15 min in a seated position, blood was drawn from an antecubital vein, centrifuged, and stored at -80°C until analysis. Serum AMH was quantified using an ultrasensitive AMH/MIS ELISA, AL-105-i (Ansh Labs, Webster, TX, USA), with intra-assay CV $<2.0\%$ and inter-assay CV 2–4%.

Statistical analysis

Statistical analysis was performed using SAS Enterprise Guide 7.1 (SAS Institute Inc., Cary, NC, USA). A power calculation declared 60 ovaries needed for 80% power to find a 1.7 mL difference in ovarian volume between modalities. A difference of 1.7 mL was chosen since the only study comparing ovarian volumes from TVUS and 3D MRI found a mean difference of 1.7 mL (12). Distribution of data was checked using histograms and probability plots. Normally distributed data are presented as mean (SD) and non-normally distributed data as median (range). Paired t-tests were performed to assess mean differences in ovarian volume and AFC, between modalities. Mean difference and 95% limits of agreement (± 1.96 SD) between MRI and 2D TVUS are presented in Bland–Altman plots (18). Similar plots were constructed with 3D TVUS data as well as with logarithmic transformed AFC data. Percentage differences were calculated from logarithmic numbers. Correlations were determined with Spearman's correlation coefficient and a multiple regression analysis with AFC from the different modalities as dependent variable and serum AMH, BMI, and age as covariates was performed.

For estimation of intra-observer reliability, ten randomly selected 3D datasets and ten MRI datasets were re-evaluated at least two months from first analysis. Intra-observer reliability was determined using intraclass correlation coefficients (ICC 2,1) from a two-way random effects ANOVA (absolute agreement) performed with IBM SPSS Statistics 22 (IBM, Armonk, NY, USA). Due to dependency of the volume and AFC of the ovaries within one subject, analyzing both ovaries of each woman would result in an incorrect doubling of the sample size. This would lead to underestimation of variances of the outcomes and thereby affect statistical tests and confidence intervals, therefore every second woman included contributed with her left ovary and every other with her right ovary. If the ovary was

missing or contained a corpus luteum or larger follicle the contralateral ovary was chosen for analysis.

Results

Sixty-six women were included (Fig. 2); 95% were Caucasian with a median age of 29 years (range, 19–44 years), mean BMI 32.7 (4.5) kg/m², and median AMH 69.8 pmol/L (range, 9.3–260.5 pmol/L). Of the women, 41% fulfilled all three Rotterdam criteria, 1% had oligo/anovulation + hyperandrogenism, 35% had oligo/anovulation + PCO, and 23% had hyperandrogenism + PCO. In total, 129 ovaries were identified with TVUS, 132 with MRI, and 66 paired observations (i.e. ovaries) were used for analysis. Due to technical problems, AFC data from 3D TVUS were missing in four cases.

Ovarian volume

Mean (SD) ovarian volume was 9.40 (3.62) mL by 2D TVUS, 10.88 (3.70) mL by 3D TVUS, and 10.65 (4.09) mL by MRI. Volume estimates from 2D TVUS were 11.6% (95% CI, 6.0–16.9; $P < 0.001$) smaller than MRI estimates and 14.9% (95% CI, 10.1–19.4; $P < 0.001$) smaller than 3D TVUS estimates (Table 1, Fig. 3). Analyses, excluding ovaries not fulfilling the PCO criteria ($n = 61$) gave analogous results. Intra-observer ICC for 3D TVUS and MRI were 0.957 (0.888–0.984) and 0.952 (0.862–0.982), respectively.

Antral follicle count

Median (range) AFC was 26.5 (4–64) by 2D TVUS, 29 (7–97) by 3D TVUS, and 29 (4–98) by MRI. Bland–Altman plots showed increasing differences with increasing AFC for all comparisons (Fig. 4). Bland–Altman statistics performed on logarithmic transformed data showed that 2D TVUS estimates were 18% (95% CI, 13–23; $P < 0.005$) smaller than 3D TVUS estimates and 16% (95% CI, 6–25; $P < 0.005$) smaller than MRI estimates (Table 1). Analyses, excluding ovaries not fulfilling the PCO criteria ($n = 58$) gave analogous results. For AFC, intra-observer ICCs for 3D TVUS and MRI were 0.987 (0.966–0.995) and 0.691 (0.026–0.897), respectively. Serum AMH correlated with AFC from 2D TVUS, 3D TVUS, and MRI, $r = 0.67$, 0.78, and 0.70 ($P < 0.001$ for all), respectively. The β -coefficients from multiple linear regression analyses with AMH, BMI, and age as covariates were 0.13 (95% CI, 0.09–0.17; $P < 0.001$), 0.22 (95% CI, 0.17–0.28; $P < 0.001$), and 0.18 (95% CI, 0.11–0.25; $P < 0.001$) for AFC from 2D TVUS, 3D TVUS, and MRI, respectively. BMI did not have a statistically significant effect on the results, but was kept in the model.

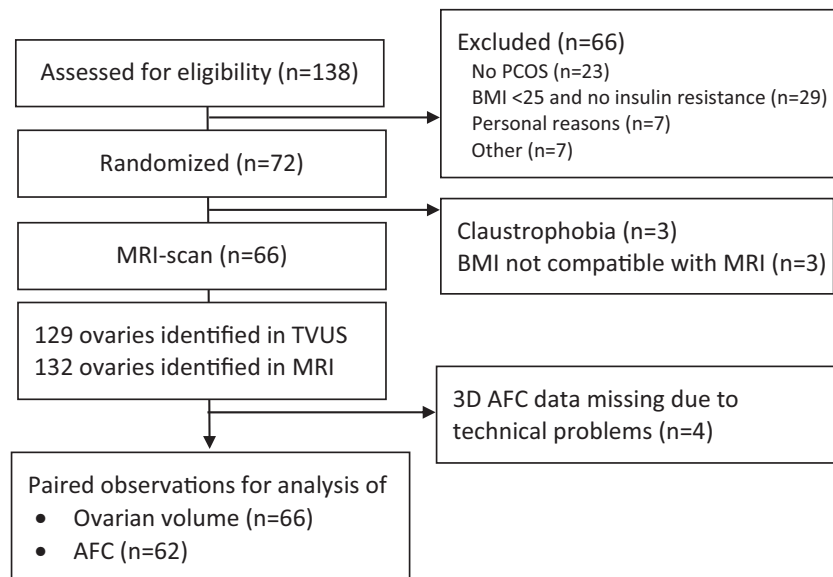


Fig. 2. Participant flow with regard to collection of TVUS and MRI data.

AFC, antral follicle count; BMI, body mass index; MRI, magnetic resonance imaging; PCOS, polycystic ovary syndrome; TVUS, transvaginal ultrasound.

Table 1. Differences in estimates of ovarian volume and AFC.

Ovarian volume (n = 66)			
	Difference (mL)	95% CI	Limits of agreement (mL)
MRI-2D	1.25*	0.62–1.87	(–3.84 – 6.33)
MRI-3D	–0.24	–0.78 – 0.31	(–4.65 – 4.18)
2D-3D	–1.48*	–2.03 – –0.94	(–5.84 – 2.87)
	Ratio	95% CI	Limits of agreement
2D/MRI	0.88*	0.83–0.94	0.54–1.45
3D/MRI	1.04	0.98–1.10	0.67–1.62
2D/3D	0.85*	0.80–0.90	0.55–1.32
AFC (n = 62)			
	Difference	95% CI	
MRI-2D	6.39*	2.44–10.34	
MRI-3D	–0.31	–4.21 – 3.60	
2D-3D	–6.69*	–8.96 – –4.43	
	Ratio	95% CI	Limits of agreement
2D/MRI	0.84*	0.75–0.94	0.35–1.98
3D/MRI	1.03	0.92–1.14	0.45–2.33
2D/3D	0.82*	0.77–0.87	0.51–1.30

Mean differences are determined using a paired t-test and ratios are assessed from logarithmic transformed data. Data are presented as mean with 95% CI (± 1.96 SEM) and limits of agreement (± 1.96 SD).

* $P < 0.005$.

2D, two-dimensional transvaginal ultrasound; 3D, three-dimensional transvaginal ultrasound; AFC, antral follicle count; CI, confidence interval; MRI, magnetic resonance imaging; SD, standard deviation; SEM, standard error of the mean.

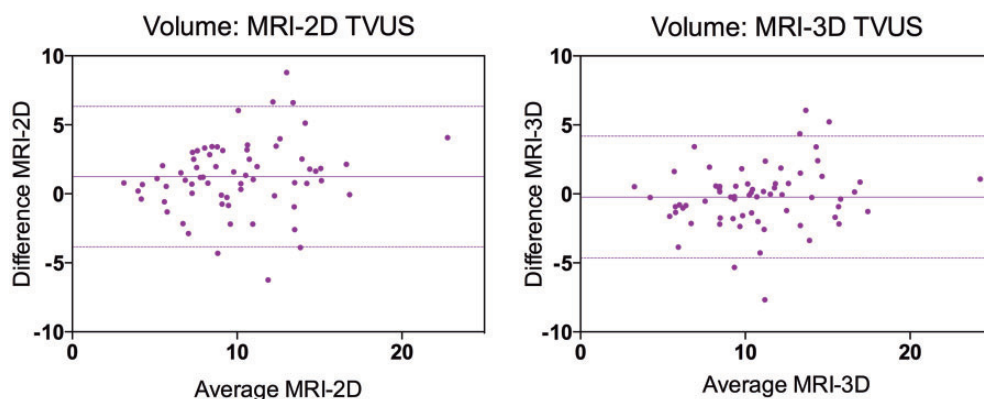


Fig. 3. Difference in ovarian volume. Bland–Altman plots showing mean (x-axis) and difference (y-axis) in ovarian volume between modalities. Full line marks mean difference and dashed lines mark 95% limits of agreement (± 1.96 SD). MRI, magnetic resonance imaging; TVUS, transvaginal ultrasound.

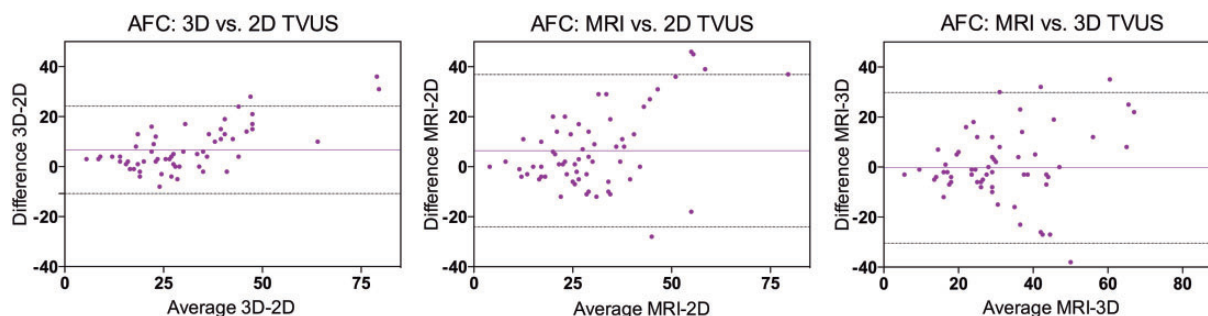


Fig. 4. Difference in antral follicle count. Bland–Altman plots showing mean (x-axis) and difference (y-axis) in antral follicle count between modalities. Full line marks mean difference and dashed lines mark 95% limits of agreement (± 1.96 SD). AFC, antral follicle count; MRI, magnetic resonance imaging; TVUS, transvaginal ultrasound.

Rotterdam criteria

With 2D TVUS, 60 of the 66 women (91%) had polycystic ovaries according to the Rotterdam ultrasound criteria (≥ 12 follicles [size range, 2–9 mm and/or volume > 10 mL]). Corresponding numbers for 3D TVUS were 63 out of 66 (95%).

Discussion

In this cross-sectional study on 66 women with PCOS, we found 2D TVUS to underestimate ovarian volume and AFC compared with 3D TVUS and MRI, whereas estimates from 3D TVUS and MRI were similar.

The 3D approach seems to give more accurate and consistent volume estimates than 2D TVUS, illustrated by narrower limits of agreement when compared to MRI. To our knowledge, two studies have previously compared TVUS and MRI with respect to ovarian morphology (12,19) with conflicting results. In a

mixed population ($n = 99$) volume estimates from 2D TVUS were 1.7 mL larger than from MRI and in 33 adolescents with PCOS, estimates from 2D US were smaller than from MRI (11.9 versus 8.8 mL) (19). Since the second study used either transvaginal or transabdominal US, results are difficult to interpret. One study found 0.33 mL smaller ovarian volume from 2D than 3D TVUS in 89 non-PCOS women (8), while others found no difference in a lean PCOS population ($n = 112$) (13) or in women undergoing in vitro fertilization treatment ($n = 20$) (20). 2D TVUS also seems to underestimate ovarian volume as compared with the true gold standard. In a study on 46 oophorectomy specimens volume estimates were 4.0 mL and 5.1 mL from 2D- and 3D TVUS, respectively, compared with 5.5 mL from Archimedes' principle (6). Accordant with this, another study found 27% smaller volumes from 2D TVUS than from Archimedes' principle (7). However, the results might be influenced by the mean time between TVUS and oophorectomy (27 days; range, 1–141 days) and the fact that volumes were calculated

based on the weight of the specimens and an ovarian tissue density calculated from Archimedes' principle from 11 cases. True volume estimates were higher than estimates from TVUS, despite the fact that post-operative specimens were non-circulated (6,7).

While 2D TVUS assumes an ovoid shape of the ovary, the 3D modalities (TVUS and MRI) outline the contours of the ovary allowing a more precise estimation in irregularly shaped ovaries, as does the Archimedes' principle.

AFC from 2D TVUS was 18% and 16% smaller than estimates from 3D TVUS and MRI, respectively. There was no difference between 3D TVUS and MRI, suggesting that 3D TVUS gives more accurate estimates of AFC than 2D TVUS. Narrower limits of agreement between 2D and 3D TVUS, than between 2D TVUS and MRI and between 3D TVUS and MRI suggest 2D and 3D TVUS are more consistent than 3D TVUS and MRI; probably because the TVUS modalities are more alike than the TVUS and the MRI. Diverging from our results, higher mean AFC was found with MRI (37.9 ± 20.3) than with 3D TVUS (SonoAVC) (23.6 ± 14.9) in 99 women with and without PCOS (12). This is probably because all 1–22 mm follicles were counted and follicles <2 mm can be difficult to distinguish in TVUS. In contrast to us, Deb et al. found higher AFC with 2D than with 3D TVUS (SonoAVC) in two studies with non-PCOS women (14,21). The second study used 3D TVUS images for 2D estimates, which may have resulted in overestimation of AFC compared with real-time 2D TVUS. The lower AFC in 3D TVUS (SonoAVC) may be due to color-coding of follicles, which prevents double counting thereby giving more accurate estimates. Post-processing of 3D data is important; Ignoring it leads to underestimation of AFC (21), which might be the reason why no difference in AFC was found between 2D and 3D TVUS in one of the largest studies comparing the modalities ($n = 164$) (13). Since different software is used for 3D AFC estimates and some authors scroll through offline 3D data for 2D AFC estimates, studies are not easily comparable. Moreover, many studies use estimates from both ovaries and as these observations are dependent the variance decreases, possibly causing inaccurate statistic conclusions.

Most studies examined normal populations or women undergoing assisted reproduction therapy. The risk of underestimating AFC is higher in ovaries with many follicles, which might explain why we, in a PCOS population, find wider limits of agreement than others. Studies evaluating AFC from 3D TVUS in non-PCOS populations present high inter-observer ICC, but decreasing ICC and wider limits of agreement with higher AFC (15,16,22). Our intra-observer ICCs for 3D TVUS was comparable with others' and the inter-observer variation was eliminated as one observer performed all TVUS.

Serum AMH was highly correlated with AFC from all three modalities and results are comparable with previously reported correlations between AMH and AFC by 3D TVUS $r = 0.75$ (follicle size, 2–9 mm) (23), 2D TVUS $r = 0.627$ (2–9 mm) (24), and MRI $r = 0.84$ (1–9 mm) (25).

This study has several strengths: a fairly large population, proper 3D software, and independent observations. However, there are some limitations. Adipose tissue is associated with poor image quality and the high BMI (mean 32.7 kg/m^2) might have reduced overall image quality. Ideally examinations are performed in early follicular phase, which is difficult in an oligo/amenoreic population. It could be argued that this is without consequence as we are comparing modalities rather than describing a population.

In conclusion, 2D TVUS underestimated ovarian volume and AFC compared with 3D TVUS and MRI, in an overweight PCOS population. Moreover, serum AMH had higher correlation with AFC from 3D than from 2D TVUS. The advantage of 3D TVUS might be of minor clinical importance when diagnosing PCOS, but useful when the actual AFC is of interest, e.g. in fertility counseling and research.

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Liraglutide in Polycystic Ovary Syndrome: A double-blind, placebo-controlled, randomized clinical trial on bleeding ratio, ovarian morphology, Anti-Müllerian hormone and sex steroid levels

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ABSTRACT

Polycystic ovary syndrome (PCOS) encompasses both an ovarian and a metabolic dysfunction. Intervention with glucagonlike peptide-1 (GLP-1) analogs facilitates weight loss and ameliorates metabolic dysfunction in overweight women with PCOS, but the effect of GLP-1 analogs on the ovarian dysfunction is only scarcely reported. In a double-blind, randomized trial 72 women with PCOS, fulfilling the Rotterdam criteria, were allocated to intervention with the GLP-1 analog liraglutide or placebo (1.8 mg/day), in a 2:1 ratio. At baseline and 26-week follow-up bleeding pattern, levels of anti-Müllerian hormone, sex hormones and gonadotropins were assessed and ovarian morphology was evaluated, using 3D-transvaginal ultrasound. Liraglutide caused 5.2 kg (95%CI 3.0-7.5, $p<0.0001$) weight loss compared with placebo. Bleeding ratio improved with liraglutide: 0.28 (95%CI 0.20-0.36, $p<0.001$) and placebo: 0.14 (95%CI 0.02-0.26, $p=0.03$), with a between group difference of 0.13 (95%CI 0.02-0.24, $p=0.02$). Free testosterone levels decreased by 18% (95%CI -1-33, $p=0.06$) and there was a trend towards reduced ovarian volume, -1.57 ml (95%CI -3.30-0.17, $p=0.08$) with liraglutide, as compared with placebo. Nausea and constipation were more prevalent in the liraglutide group. Liraglutide improved markers of ovarian function in an overweight PCOS population and might be an alternative to existing intervention possibilities in treating PCOS.

Abbreviations

3D, three-dimensional; AMH, anti-Müllerian hormone; COC, combined oral contraceptives; FG, Ferriman Gallway; GLP-1, glucagonlike peptide-1; HOMA2-IR, Homeostasis model assessment-estimated insulin resistance; PCOS, polycystic ovary syndrome; TVUS, transvaginal ultrasound

INTRODUCTION

With a prevalence of 10% polycystic ovary syndrome (PCOS) is a common endocrine disorder in premenopausal women (Bozdag *et al.* 2016). The syndrome has a complex pathophysiology with two main issues: a metabolic and an ovarian dysfunction, seen isolated or simultaneously (Dunaif and Fauser 2013). Central for PCOS is the ovarian dysfunction, clinically seen as oligo/anovulation, androgen excess and polycystic ovarian morphology. These three represent the Rotterdam criteria upon which the PCOS diagnosis is based (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group 2004).

The folliculogenesis is a highly complex process directed by auto-, para- and endocrine factors, e.g. gonadotropins, sex steroids and anti-Müllerian hormone (AMH) (Dewailly *et al.* 2016). The glycoprotein AMH is produced by the granulosa cells of early developing follicles (Pellatt *et al.* 2007). By inhibiting follicular recruitment and FSH-dependent follicular growth AMH is thought to have a protective effect on the ovarian reserve in the healthy individual (Dumont *et al.* 2015). Due to a large pool of early developing follicles and greater AMH production per follicle women with PCOS have higher AMH levels than controls (Pellatt *et al.* 2007, Piouka *et al.* 2009) and the protective mechanism goes beyond its physiological purpose, causing "follicular arrest" and anovulation (Dumont *et al.* 2015). In overweight women with PCOS weight loss recovers the arrested folliculogenesis seen as increased ovulation rate and improved bleeding pattern (Nybacka *et al.* 2013), whereas metformin, possibly through increased insulin sensitivity, improves cycle regularity in both over- and normal weight women with PCOS (Romualdi *et al.* 2010, 2011). Indeed, insulin is a central factor in PCOS. Intrinsic and obesity-related insulin resistance causes compensatory hyperinsulinemia, which amplifies the excessive LH-stimulated ovarian androgen production (Tosi *et al.* 2012) and decreases the hepatic SHBG production, ultimately causing hyperandrogenism (Nestler *et al.* 1991).

The ideal PCOS treatment addresses both the metabolic and the ovarian dysfunction of the syndrome. Over the last decade glucagon-like peptide 1 (GLP-1) analogs are increasingly used in treating type 2 diabetes and obesity, where they mimic the effect of the endogenous incretin hormone, promoting weight loss, improved glycemic control and reduced dyslipidemia (Vilsbøll *et al.* 2012). Due to the resemblance between type 2 diabetes and PCOS and the fact that metformin affects the ovarian dysfunction the GLP-1 analogs might have a role in treating the syndrome. The

GLP-1 analog liraglutide has been found to cause weight loss in several small trials on women with PCOS (*Jensterle et al. 2015a, 2015b, 2015c, Kahal et al. 2015*), but results regarding bleeding frequency are sparse.

We hypothesized that intervention with a GLP-1 analog would improve the ovarian dysfunction in overweight women with PCOS, possibly through a weight loss and altered glucose metabolism. In this randomized, clinical trial we aimed to investigate the effect of liraglutide in women with PCOS on markers of ovarian dysfunction: bleeding ratio, ovarian morphology, levels of AMH and androgens.

METHODS

Protocol & ethics

This was an investigator-initiated, double blind, placebo-controlled, randomized trial, conducted from March 2014 to December 2015 at Herlev Gentofte Hospital, University of Copenhagen, Denmark. The study was approved by the regional Ethics Committee of the Capital Region of Denmark (ID: H-2-2013-142), the Danish Health Authority (EudraCT: 2013-003862-15) and the Danish Data Protection Agency. The study was conducted in accordance with Good Clinical Practice guidelines and the declaration of Helsinki. Oral and written consent were obtained for each participant prior to inclusion. The protocol has been published elsewhere (*Frøssing et al. 2015*). At baseline and 26-week follow-up, anthropometrics and Ferriman-Gallway (FG) score were assessed, blood tests, a 75 g oral glucose tolerance test and a transvaginal ultrasound were performed. Blood was drawn between 8.00-10.00 AM after overnight fasting. Blood for determination of AMH and insulin levels was centrifuged and serum was stored at -80°C until analysis.

Population

In Denmark, women with PCOS are in general managed by a general practitioner or a private-practicing gynecologist, rather than in a hospital setting. Because of this, women were mainly recruited from social media (www.facebook.com/PCOS/kliniskforsoeg), from advertising in the local area and from general practitioners or private gynecologists but also from our out-patient clinic. Briefly, inclusion criteria were: age >18 years, PCOS according to Rotterdam criteria (*Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group 2004*), BMI ≥ 25 kg/m² and/or insulin resistance (defined as fasting plasma C-peptide >600 pmol/L). Irregular bleeding was defined as cycle length >35 days and hyperandrogenism as total and/or free testosterone above reference levels (1.80 nmol/L and 0.0034 nmol/L, respectively) and/or FG score ≥ 8 . In brief, exclusion criteria were: pregnancy, diabetes, use of hormonal contraceptives (within six weeks prior to inclusion), anti-diabetic and/or anti-androgenic agents (within three months prior to inclusion). Other causes of irregular menstruation and androgen excess, e.g. hyperprolactinemia, thyroid and adrenal diseases were excluded, using biochemical work-up performed at screening.

Randomization & intervention

Women were randomized to 26 weeks of intervention with liraglutide or placebo in a 2:1 ratio, in blocks of six. Novo Nordisk A/S, Bagsværd, Denmark provided identically packed and labeled study drug (liraglutide and placebo) as well as a randomization list. All participants and investigators were blinded as an independent secretary handled the randomization list and instructed the investigators in which serial numbers (i.e. drug packages) to

be handed out to each participant. The study drug was administered subcutaneously 1.8 mg/day, starting at 0.6 mg/day and 1.2 mg/day for the first and second week, respectively.

Outcomes

Pre-specified outcomes included the between-group difference in bleeding ratio at follow-up, as well as the between-group differences in change (from baseline to follow-up) in bleeding ratio, ovarian volume, stromal volume, antral follicle count (AFC), FG score, serum levels of AMH, SHBG and androgens.

Antroprometrics, bleeding & ultrasound

Body weight was assessed in light clothing, after overnight fasting. Waist circumference was measured by a single observer and in a standardized way: Halfway between 12th rib and the anterior superior iliac spine. At screening and baseline participants were questioned regarding number of menstrual bleedings for the last six months. The women were asked to keep a bleeding diary during the study. Bleeding ratio was defined as number of menstrual bleedings divided by study period (months). A bleeding ratio of 1.0 corresponds to six menses in six months, i.e. a cycle length of 30.4 days. A cycle length of 35 days corresponds to a bleeding ratio of 0.87 ((365 days/35 days/month)/12 months = 0.87). Transvaginal 3D ultrasound was performed by a single investigator (MN) using a Voluson E6, GE Healthcare, Chicago, IL, USA. Data were analyzed with the VOCAL and SonoAVC-tools, 4DView, GE Healthcare, Chicago, IL, USA. Follicles 2-9 mm were counted and stromal volume was calculated as ovarian volume - follicular volume. Intra-observer intra class correlations for volume and AFC were 0.957 and 0.987, respectively.

Assays

All serum AMH samples were measured in the same run, using an AL-105-i ultra-sensitive AMH/MIS ELISA (Ansh Labs, Webster, TX, USA) with intra-assay CV <2.0%. Serum levels of LH, FSH and estradiol were determined using an ADVIA Centaur Immunoassay (Siemens Healthcare GmbH, Erlangen, Germany) with inter-assay CV <3.8%, <3.9% and <10.2%, respectively. Testosterone, androstenedione, DHEAS and 17-OH progesterone levels were determined using liquid chromatography and double mass spectrometry (UPLC-MSMS TQ-S System, Waters Corporation, Milford, MA, USA), with inter-assay CV $\leq 12\%$ for all. SHBG levels were measured using a sandwich chemiluminescence immunometric method (Immulite 2000, Siemens Healthcare GmbH, Erlangen, Germany) with inter-assay CV <7%. Free testosterone (*Bartsch 1980*) and free androgen index (total testosterone x 100/SHBG) were calculated from total testosterone and SHBG. Fasting plasma glucose and HbA1c levels were assessed using in-house routine analyses. Plasma insulin levels were determined with an electrochemiluminescence immunoassay and Cobas e422 reader (Roche Diagnostics GmbH, Mannheim, Germany) with intra-assay CV 2.1%. An online calculator (www.dtu.ox.ac.uk/homacalculator) was used calculating the Homeostasis model assessment-estimated insulin resistance (HOMA2-IR) from fasting insulin and fasting glucose.

Statistics

Distribution of data was evaluated using histograms and qq-plots. Non-normally distributed data were subject to logarithmic transformation. Data are presented as mean (SD), median (p25-p75) and mean differences (95% confidence interval (CI)). Difference from baseline to follow-up in each group was estimated with a

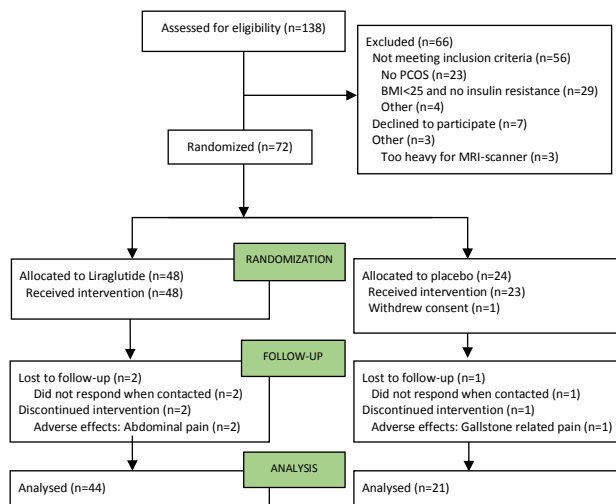


Figure 1. Participant flow

paired t-test and between-group difference in effect size was calculated using a linear mixed model with repeated measurements and maximum likelihood. Since some of the data were logarithmically transformed for the mixed model some effect sizes are presented as ratios. Every other woman contributed with her right ovary and every second with her left for ovarian morphology outcomes. Associations between change in bleeding ratio and baseline bleeding ratio, as well as change in anthropometric, endocrine, metabolic and ovarian morphology variables were assessed using Pearson's or Spearman's correlation coefficients, as appropriate, and further analyzed using multiple linear regression analyses. This study was a part of a trial investigating the effect of liraglutide on thrombin generation, where a sample size calculation stated 63 women randomized in a 2:1 ratio needed. For the outcome, between-group difference in change in bleeding ratio, with 63 women, a standard deviation of 0.33 and a significance level of 0.05 the study had 95% power to find an effect size of 0.33. This effect size corresponds to two menstruations/six months and is supported by results from a previous study, using exenatide in oligo/anovulatory PCOS (Elkind-Hirsch *et al.* 2008). In a population with an-/oligo amenorrhea Elkind-Hirsch *et al.* found a SD of 0.1. However, by using the Rotterdam criteria we will include women with regular menstruations and our SD will be higher, therefore 0.33 was chosen. Seventy-two women were included to allow for drop-outs. Analyses were performed using SAS Enterprise Guide 7.1, SAS 9.4, SAS Inc., Cary, NC, USA.

RESULTS

Of 138 women assessed for eligibility, 72 were randomized and 65 completed the trial (Figure 1, Table I). In the placebo group one woman dropped out at the day of randomization, due to personal reasons. Apart from this, reasons for dropout were similar in the two groups: lost to follow-up (4.2%) and abdominal pain (4.2%). At baseline there were no differences in bleeding ratio, ovarian morphology, levels of AMH, sex hormones or SHBG between groups. Even though not statistically significant, the liraglutide group appeared older and with lower proportion of smokers and between-group differences where adjusted for age, BMI and smoking status.

After six months, the liraglutide group had a mean weight loss of 5.2 kg (95%CI 3.0-7.5, $p<0.0001$) and mean reductions in fasting

Table 1. Baseline

	LIRAGLUTIDE (N=48)	PLACEBO (N=24)
Age (years)	31.4 (24.6-35.6)	26.2 (24.8-31.5)
Weight (kg)	94.2 (15.4)	91.3 (13.6)
BMI (kg/m ²)	33.3 (5.1)	33.3 (4.6)
Waist/hip ratio	0.91 (0.08)	0.92 (0.10)
Pregnancies (full born)	0 (0-2)	0 (0-1)
Smoking (<10 cigarettes/day)	18.8% (9)	33.3% (8)
Ethnicity		
Caucasian	93.8% (45)	91.7% (22)
Rotterdam phenotype		
OA + HA + PCO	39.6% (19)	45.8% (11)
OA + HA	2.1% (1)	8.3% (2)
HA + PCO	22.9% (11)	16.7% (4)
OA + PCO	35.4% (17)	29.2% (7)
Amenorrhea /Oligomenorrhea /Regular menstruation	23%/60%/17% (8/29/11)	21%/58%/21% (5/14/5)
Systolic (mmHg)	123 (9)	124 (9)
Diastolic (mmHg)	79 (8)	80 (7)
HbA1c (mmol/mol)	34.2 (2.8)	34.6 (3.4)
HOMA2-IR	2.29 (1.83-2.84)	2.42 (1.91-3.20)
GFR (ml/min)	112.8 (13.3)	117.5 (9.7)

Results presented as mean (SD), median (p25 - p75) or percentage (n).

Differences at baseline assessed with unpaired t-test, Mann-Whitney U-test, Chi²-test or Fishers exact test, as appropriate. BMI, body mass index; OA, oligo-/amenorrhea; HA, hyperandrogenism; PCO, polycystic ovaries; HbA1c, glycosylated hemoglobin; HOMA2-IR, Homeostasis model assessment-estimated insulin resistance; GFR, glomerular filtration rate.

glucose and HbA1c of 0.24 mM (95%CI 0.05-0.43, $p<0.05$) and 1.4 mmol/mol (95%CI 0.3-2.5, $p<0.05$), respectively, compared with the placebo group. We observed no effect on fasting insulin or HOMA2-IR (data not shown).

Bleeding

At follow-up 62% (26 of 42) of women in the liraglutide group had a bleeding ratio >0.87 and corresponding number in the placebo group was 28% (5 of 18) (difference between groups, $p<0.05$). At baseline the number of women with amenorrhea, oligomenorrhea and regular menstrual bleedings were 8, 29 and 11 ($n=48$) in the liraglutide group and 5, 14 and 5 ($n=24$) in the placebo group. At follow-up corresponding numbers were 2, 14 and 26 ($n=42$), and 1, 12 and 5 ($n=18$).

Change in bleeding ratio correlated with bleeding at baseline ($r=-0.66$, $p<0.001$) and change in BMI ($r=-0.28$, $p<0.05$). There was no significant correlation between the change in bleeding ratio after intervention and the change in waist circumference, levels of androgens, gonadotropins, AMH, fasting glucose, fasting insulin or HOMA2-IR (data not shown). Excluding the 16 women with regular menstruation at baseline did not alter the results. In a multiple regression analysis with change in bleeding ratio as dependent variable both study drug and baseline bleeding ratio had significant impact. Adding change in BMI to the model, the study drug-effect became non-significant, whereas adding change in HOMA2-IR did not alter the results.

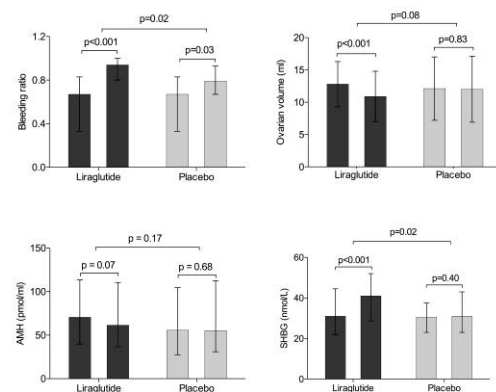


Figure 2. Baseline and follow-up: Bleeding ratio, ovarian volume and levels of AMH and SHBG. Data are presented as median (p25-p75). Ovarian volume data are presented as mean (SD). AMH, anti-Müllerian hormone; SHBG, sex hormone binding globulin.

Table 2. Table II. Baseline values and changes in outcome measures from baseline to week 26 during liraglutide or placebo treatment

	LIRAGLUTIDE			PLACEBO			DIFFERENCE BETWEEN GROUPS			
	Baseline (n=48)	Difference (n=44)	p	Baseline (n=24)	Difference (n=21)	p	Crude	p	Adjusted ^d	p
Bleeding ratio	0.67 (0.33-0.83)	0.28 (0.20-0.36) ^b	<0.001	0.58 (0.33-0.83)	0.14 (0.02-0.26) ^c	0.03	0.14 (0.03-0.24)	0.01	0.13 (0.02-0.24) ^b	0.02
Ovarian volume (ml)	12.8 (3.5) ^a	-2.0 (-3.1 - -0.9) ^a	<0.001	12.1 (4.9) ^a	-0.2 (-1.7-1.4) ^a	0.83	-1.6 (-3.3-0.1)	0.07	-1.57 (-3.30-0.17)	0.08
Antral follicle count	29.0 (22.5-44.0) ^d	-2.0 (-6.0-2.0) ^b	0.31	28.0 (16.0-43.0) ^b	2.5 (-2.0-7.0)	0.25	0.88 (0.74-1.06) ^e	0.17	0.90 (0.75-1.08) ^e	0.23
Stroma volume (ml)	11.4 (2.9) ^d	-1.9 (-3.1 - -0.8) ^b	<0.01	10.7 (4.5) ^b	-0.2 (-1.7-1.2)	0.77	0.86 (0.71-1.03) ^e	0.09	0.86 (0.72-1.03) ^e	0.10
AMH (pmol/ml)	70.5 (39.7-113.4)	-8.4 (-17.4-0.6)	0.07	72.3 (27.5-104.7) ^a	3.5 (-13.9-21.0) ^a	0.68	0.87 (0.72-1.04) ^e	0.13	0.88 (0.74-1.06) ^e	0.17
LH (IU/L)	8.0 (5.1-12.9)	-1.7 (-5.9-2.6)	0.43	8.7 (4.5-14.2)	1.0 (-2.7-4.6)	0.59	1.08 (0.73-1.59) ^e	0.70	1.12 (0.77-1.63) ^e	0.54
FSH (IU/L)	6.1 (3.8-7.9)	-0.3 (-1.3-0.8)	0.64	5.8 (4.6-6.6)	0.2 (-1.3-1.7)	0.76	0.95 (0.74-1.21) ^e	0.68	0.95 (0.74-1.22) ^e	0.69
Estradiol (nmol/L)	0.25 (0.17-0.58)	-0.04 (-0.07-0.14)	0.49	0.24 (0.19-0.39)	0.02 (-0.14-0.11)	0.80	1.01 (0.74-1.39) ^e	0.94	1.00 (0.73-1.37) ^e	0.98
Total testo. (nmol/L)	1.23 (0.91-1.63)	-0.07 (-0.25-0.10)	0.41	1.35 (0.95-1.93)	0.15 (-0.10-0.39)	0.23	0.88 (0.71-1.09) ^e	0.26	0.90 (0.73-1.10) ^e	0.30
Free testo. (nmol/L)	0.026 (0.021-0.038)	-0.005 (-0.009 - -0.001)	0.01	0.033 (0.023-0.040)	0.004 (-0.003-0.011)	0.22	0.81 (0.65-1.00) ^e	0.05	0.82 (0.67-1.01) ^e	0.06
Free androgen index	3.84 (2.78-6.54)	-1.34 (-2.19 - -0.48)	<0.01	4.95 (3.08-6.32)	0.80 (-0.42-2.01)	0.18	0.74 (0.58-0.95) ^e	0.02	0.76 (0.61-0.96) ^e	0.02
Androst. (nmol/L)	6.31 (4.39-7.93) ^a	-0.69 (-1.44-0.06)	0.07	6.29 (4.63-8.84)	0.76 (-0.39-1.92)	0.18	0.85 (0.70-1.04) ^e	0.11	0.88 (0.73-1.07) ^e	0.20
SHBG (nmol/L)	31.0 (22.0-44.5)	7.4 (4.1-10.7)	<0.001	30.5 (23.0-37.5)	2.0 (-2.9-7.0)	0.40	1.19 (1.02-1.39) ^e	0.03	1.18 (1.03-1.36) ^e	0.02

Data presented as mean (SD), median (p25-p75) and differences as mean (95%CI). Missing: ^a= one, ^b= two, ^c= three, ^d= four. ^e= presented as a ratio.

^fExclusion of the 16 women with regular menstruation at baseline did not significantly alter the results.

^gMixed model adjusted for age, BMI and smoking status at baseline. AMH, anti-Müllerian hormone; LH, luteinizing hormone; SHBG, Sex hormone binding globulin.

Ovarian morphology & endocrine markers

In the liraglutide group, ovarian and stromal volume decreased by 16% (95%CI 7-24%, $p<0.001$) and 17% (95%CI 7-27%, $p<0.01$), respectively and there was a drift towards reduced volume when compared with the placebo group ($p=0.08$ and $p=0.10$, respectively, Table II). There was a trend towards decreased serum AMH in the liraglutide group, -8.4 pmol/ml (95%CI -17.4-0.6, $p=0.07$), but no difference between groups (Figure 2). We observed no effect on FG score in either group. Results regarding endocrine markers are presented in Table II.

Adverse effects

The most prevalent adverse effects were nausea (liraglutide 79% vs. placebo 13%, $p<0.01$), primarily seen in the startup phase, and constipation (26% vs. 0%, $p<0.01$). Gallstone-related pain was experienced by 6% of women in the liraglutide group and 4% in the placebo group (NS). Two of the women in the liraglutide group had a cholecystectomy and due to discontinuation of the study drug during diagnosis and surgery their overall compliance was 64% and 87%, respectively, and both were included in final analyses.

DISCUSSION

In this randomized, placebo-controlled trial investigating the effect of the GLP-1 analog liraglutide on the ovarian dysfunction in PCOS, we found improved bleeding ratio, decreased free testosterone and a trend towards lower ovarian and stromal volume.

Accordant with our findings, *Elkind-Hirsch et al.* found improved bleeding pattern with the GLP-1 analog exenatide. They treated 42 overweight women with oligo-ovulatory PCOS with exenatide, metformin or a combination for 24 weeks and found improved ovulation rate in all groups, with the combined treatment being superior (*Elkind-Hirsch et al. 2008*). Additionally, they found reduced body weight and insulin resistance, as measured by the *Homeostasis model assessment-estimated insulin resistance* (HOMA-IR). Contrary to our results, several papers on liraglutide in PCOS find unaltered bleeding frequency despite an effect on body weight (*Jensterle et al. 2015a, 2015b*) and HOMA-IR (*Kahal et al. 2015*). This is probably due to small sample size (*Kahal et al. 2015*), short duration (*Jensterle et al. 2015a, 2015b*) and low liraglutide dose (*Jensterle et al. 2015a, 2015b*).

Weight loss is known to cause improved bleeding pattern in overweight women with PCOS, found in life-style intervention studies (*Moran et al. 2007, Nybacka et al. 2011, Thomson et al. 2009*) and after bariatric surgery (*Turkmen et al. 2016*). *Moran et al.* proposed reduced insulin resistance to be the mechanism for this improved bleeding pattern, as women, experiencing improved bleeding with weight loss had reduced fasting insulin levels, compared with non-responders (*Moran et al. 2007*). This theory is supported by the fact that metformin treatment improves bleeding pattern in normal- and overweight women with PCOS (*Elkind-Hirsch et al. 2008, Romualdi et al. 2010, 2011*). Both *Nybacka et al.* and *Thomson et al.* found improved bleeding regularity with lifestyle-induced weight loss (3-11%), but no alterations in HOMA-IR, in overweight women with PCOS (*Nybacka et al. 2011, Thomson et al. 2009*). We found reduced fasting glucose and HbA1c, indicating improved insulin sensitivity, in the liraglutide group as compared with the placebo group. However, we could not demonstrate this by a decrease in HOMA2-IR. Additionally, we found the improved bleeding ratio to be driven by weight loss rather than by changes in HOMA2-IR or by at direct effect of liraglutide. Most likely, both weight loss and subtle alterations in insulin levels play a role in restoring the bleeding pattern. We found increased SHBG levels, decreased free testosterone levels and unaltered total testosterone levels with 26 weeks of liraglutide treatment. Corroborating this, liraglutide caused increased SHBG levels and unaltered total testosterone levels in a 12-week study comparing liraglutide with the combination of liraglutide and metformin in obese women with PCOS (*Jensterle et al. 2016*). Inconsistent with our results, 24 weeks of exenatide was found to cause reduced total testosterone levels and unaltered SHBG levels, in a population with higher baseline testosterone levels than ours (*Elkind-Hirsch et al. 2008*). In open-labelled studies with smaller populations (*Kahal et al. 2015*), shorter follow-up (12 weeks) and lower liraglutide dose (1.2 mg/day) (*Jensterle et al. 2015a, 2015b, 2015c*) liraglutide was found to have no effect on either total testosterone or SHBG levels. Ovarian stroma is highly correlated with androgen levels (*Fulghesu et al. 2007*) and the trend observed in our study, towards decreased stroma volume, may indicate a reduced ovarian androgen production with liraglutide.

We did not observe any effect on FG score, most likely due to too short follow-up for this parameter. Since combined oral contraceptives (COC) effectively raise SHBG (*Mes-Krowinkel et al. 2014*), thereby decreasing the levels of free testosterone the effect of

discontinuation of COC could counteract the effect of liraglutide even though we applied a well-accepted wash-out period of at least six weeks. However, the percentage of women who recently discontinued these drugs was similar between the two groups and in sub-analyses, excluding women who discontinued COC, metformin or spironolactone therapy less than five months prior to enrollment, results, regarding SHBG, androgens, hirsutism and ovarian morphology were unaltered.

The effect of GLP-1 analogs on AMH has, to our knowledge, never been investigated before. We observed a trend towards decreased AMH over time in the liraglutide group, but no between-group difference. Decreased AMH levels have been observed together with improved bleeding frequency in women with PCOS after bariatric surgery (Bhandari *et al.* 2016) and diet-induced weight loss (Nybacka *et al.* 2013). Nybacka *et al.* found the diet-induced reduction in AMH to be correlated to the fall in testosterone and gonadotropins rather than to the weight loss (Nybacka *et al.* 2013). Conversely, a 20-week dietary program in 52 overweight women with PCOS had no significant effect on AMH despite improved bleeding frequency, reduced body weight and total testosterone levels (Thomson *et al.* 2009). Since the bleeding ratio improved in this study, we assume that some of the AMH-producing follicles bypassed the follicular arrest and were eliminated through ovulation, thereby making the pool of AMH-producing follicles smaller, which theoretically would cause lower AMH levels and AFC. Unfortunately, this was not the case in our study, possibly due to lack of power or too short follow-up.

This study has several strengths: The study design, the high compliance and the use of the double mass spectroscopy for determination of androgen levels. Moreover, ovarian morphology was assessed with 3D ultrasound, both total and stromal volumes were analyzed and all exams were performed by a single, blinded observer (MN). However, the study has some limitations: Participants did not keep a bleeding diary prior to inclusion and the reported bleeding ratio at baseline could be associated with recall bias. This could be the reason for us finding improved bleeding pattern in the placebo group. Nevertheless, there was a significant difference between the two groups demonstrating an effect of liraglutide. Since most of our participants had irregular cycles we were not able to evaluate gonadotropins in early follicular phase as appropriate, which probably have influenced on our results. Evaluating changes in the bleeding pattern in a population, including women with regular menstruation (due to the use of Rotterdam criteria) might seem inappropriate. Nevertheless, this reflects the true clinical situation and excluding the women with regular menstruation from analyses did not change the results.

In conclusion, 26 weeks of liraglutide intervention altered the ovarian dysfunction in an overweight PCOS population. It improved the bleeding ratio and reduced the free testosterone levels, most likely through weight loss and improved glucose metabolism. Of side effects nausea, especially in the up-titrating phase, and constipation was seen with liraglutide. Larger randomized studies with longer follow-up are warranted to further explore the role of liraglutide in treating PCOS.

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JF, SOS and CK initiated the study, all authors contributed to the protocol and to the intellectual interpretation of the results. SF and MN conducted the study and collected data. MN performed the statistical analyses and produced the manuscript, which all co-authors have read and approved.

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Liraglutide in Polycystic Ovary Syndrome: A double-blind randomized clinical trial investigating effects on the thrombogenic potential: Thrombin generation, fibrinolysis and low-grade inflammation

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Objective: Polycystic ovary syndrome (PCOS) is associated with increased risk of venous thromboembolism (VTE) and cardiovascular disease (CVD) in later life. We aimed to study the effect of liraglutide intervention on both VTE and CVD risk markers:

Thrombin generation, endothelial dysfunction and low-grade inflammation in overweight and/or insulin resistant women with PCOS.

Methods: In a double-blind, placebo-controlled randomized trial 72 women with PCOS were randomized, in a 2:1 ratio, to liraglutide or placebo 1.8 mg once daily for 26 weeks. Primary endpoint was between-group difference in change in endogenous thrombin potential (ETP) from baseline to follow-up, measured by thrombin generation test (TGT). Secondary endpoints were between-group differences in change in other TGT parameters (peak thrombin concentration, lag-time and time to peak), levels of von Willebrand factor (vWF), plasminogen activator inhibitor-1 (PAI-1) and high sensitivity C-reactive protein (hsCRP).

Results: Mean weight loss in the liraglutide group was 5.2 kg (95%CI 3.0-7.5 kg, $p < 0.001$) compared with placebo. We detected no effect on ETP in either group. In the liraglutide group peak thrombin concentration decreased by 16.71 nmol/L (95%CI 2.32-31.11, $p < 0.05$) and lag-time and time to peak increased by 0.13 min (95%CI 0.01-0.25, $p < 0.05$) and 0.38 min (95%CI 0.09-0.68, $p < 0.05$), respectively, while there were no between-group differences. With liraglutide PAI-1 decreased 12% (95%CI 0-23, $p = 0.05$) and there was a trend towards decreased PAI-1 when compared with placebo ($p = 0.10$).

Conclusion: In overweight women with PCOS liraglutide intervention caused an approximate 5% weight loss. In addition, liraglutide treatment affected thrombin generation, although not differently from placebo. A concomitant trend towards improved fibrinolytic activity indicates a possible reduction of the baseline thrombogenic potential. The findings point towards beneficial effects of liraglutide on markers of VTE and CVD risk, which should be further pursued in larger studies.

Abbreviations: BMI = body mass index, BP = blood pressure, O/A = oligo-/amenorrhea, HA = hyperandrogenism, PCO = polycystic ovaries, SHBG = sex hormone binding globulin, LDL = low density lipoprotein, HDL = high density lipoprotein, HbA1c = glycated hemoglobin, HOMA2-IR = homeostasis model assessment-estimated insulin resistance, GFR = glomerular filtration rate.

Introduction

With a prevalence of 10% polycystic ovary syndrome (PCOS) is the most common endocrine disorder among women of fertile age (1). The diagnosis is based on the three Rotterdam criteria: oligo-/amenorrhea, androgen excess and polycystic ovaries, where a minimum of two should be fulfilled and other etiologies excluded (2). The Rotterdam criteria encompass an ovarian dysfunction, but it is evident that PCOS also has a metabolic component, covering abdominal obesity, hypertension and dyslipidemia, as well as to insulin resistance (3). The abdominal obesity is characterized by a state of low-grade inflammation. Visceral adipose tissue secretes pro-inflammatory adipokines promoting an inflammatory state, contributing to endothelial dysfunction, which ultimately is associated with an increased risk of cardiovascular disease (CVD) (4).

PCOS is associated with both CVD and venous thromboembolism (VTE). A recent meta-analysis found odds ratio of 1.44 (1.13-1.84) for coronary heart disease in PCOS vs. non-PCOS (5) and a cohort-study including 87 000 participants found 1.5-fold higher risk for VTE in women with PCOS than in controls (6). In general, cardiovascular events are rare in premenopausal women and markers of low-grade inflammation and endothelial dysfunction often are used as measures of CVD risk in interventional studies in PCOS. Associated with atherosclerotic CVD, levels of von Willebrand factor (vWF), plasminogen activator inhibitor 1 (PAI-1) and high-sensitivity-CRP (hsCRP) are frequently used as surrogate CVD risk markers (7-9) and are all found elevated in PCOS (10,11). Elevated thrombin generation, as measured by thrombin generation test, indicates hypercoagulability, as seen in users of combined oral contraceptives (12). Moreover, high thrombin generation is associated with first and recurrent VTE and possibly with coronary atherosclerosis (13-15). Thrombin generation has been found elevated in PCOS (12), but conflicting data exist (16). In a previous study our group found high thrombin generation, PAI-1 and hsCRP to be linked to the overweight and insulin resistant PCOS phenotype (17,18). Thrombin generation seems mainly driven by overweight (19,20) and is found to decrease with weight loss (21-23). The increased thrombin generation, together with hypofibrinolysis as measured by elevated PAI-1 levels, could be a link to the increased risk of VTE in PCOS.

Lifestyle intervention and metformin are mainstays in the treatment of PCOS, aiming at reducing body weight and insulin resistance. In women with PCOS metformin has been found to reduce PAI-1 activity and levels of hsCRP to a mild degree (24) and might to some extent diminish the increased thrombin generation induced by oral contraceptives, as recently investigated (12). Glucagonlike peptide-1 (GLP-1) analogs are commonly used in type 2 diabetes and obesity, where they promote weight loss, improve glycemic control and have been demonstrated to reduce plasma levels of cholesterol, PAI-1 and hsCRP (25–27). Glucagonlike peptide-1 analogs have been used in PCOS in smaller trials, all demonstrating weight loss, but none investigating the effect on thrombin generation or fibrinolysis (28–32).

We hypothesized that intervention with the GLP-1 analog liraglutide, in overweight PCOS, would lead to a beneficial reduction in VTE and CVD risk markers: thrombin generation, vWF, PAI-1 and hsCRP, possibly due to a weight loss. Therefore, we performed a randomized clinical trial (RCT) treating women with PCOS with either liraglutide or placebo for six months.

Methods

A randomized, placebo-controlled, double-blind clinical trial conducted from March 2014 to December 2015 at Herlev Gentofte Hospital, University of Copenhagen, Denmark. Seventy-two women were randomized in a 2:1-ratio to 26 weeks of intervention with liraglutide or placebo 1.8 mg once daily. The study is registered at www.clinicaltrials.gov: NCT02073929.

Participants

Participants were enrolled from social media (www.facebook.com/PCOSkliniskforsoeg), from private practicing gynecologists and from our outpatient PCOS clinic, securing external validity. In- and exclusion criteria are published elsewhere (33). In short, eligible women were ≥ 18 years, premenopausal and had PCOS according to Rotterdam criteria, i.e. minimum two of the three 1) oligo-/amenorrhea (cycle > 35 days) 2) clinical (Ferriman Gallway score ≥ 8) or biochemical hyperandrogenism (total- or free testosterone levels above reference: > 1.8 nmol/L and > 0.034 nmol/L, respectively), and 3) polycystic ovaries (≥ 12 follicles 2–9 mm and/or volume > 10 ml in at least one ovary) on transvaginal ultrasound. Other causes to bleeding irregularities and androgen excess were excluded. The women should have BMI ≥ 25 kg/m² and/or insulin resistance defined as fasting plasma C-peptide > 0.6 nmol/L. In brief, exclusion criteria were pregnancy, breastfeeding, smoking > 10 cigarettes/day, diabetes, hypertension, overt inflammatory disease, use of herbal medicine or medications known to affect the hemostatic system. The use of hormonal contraceptives within six weeks, injectable hormonal contraceptives within six months, antidiabetic or antihypertensive drugs within three months prior to randomization led to exclusion.

Ethics

The study was approved by the Danish Data Protection Agency and the Ethics Committee of the Capital Region of Denmark (ID: H2-2013-142, EudraCT: 2013-003862-15) and performed in accordance with General Clinical Practice guidelines and the declaration of Helsinki. Participants gave oral and written informed consent prior to screening.

Intervention, randomization and blinding

Liraglutide/placebo was administered as a subcutaneous injection once daily: 0.6 mg the first week, 1.2 mg the second week and 1.8

mg for the rest of the study period (26 weeks in total). This dose was chosen as it successfully caused weight loss and improved glycemic control in obese patients with type 2 diabetes (34). The participants registered compliance daily in a medical diary provided by the study personnel. The study drug (liraglutide and placebo) was delivered in identical prefilled pens labeled with serial numbers and accompanied by a randomization list. As investigators and participants were blinded an independent secretary instructed investigators in which serial numbers to supply each woman with. The participants were randomized in a 2:1 ratio (liraglutide:placebo), as we believed that this would facilitate the recruitment to the study.

Outcomes

Primary outcome was the difference between the groups in change from baseline to follow-up in endogenous thrombin potential (ETP) measured by thrombin generation test (TGT). Secondary outcomes were differences between groups in change from baseline to follow-up in other parameters of TGT (described in Assays subsection) as well as plasma levels of vWF, PAI-1 and hsCRP.

Protocol

The women participated in four visits: Screening, baseline (week 0), safety (week 8) and follow-up (week 26), previously described (33). At screening informed consent was obtained after which a medical interview, physical exam, blood samples and transvaginal ultrasound were performed. At baseline and follow-up visits participants had blood drawn (between 8.00–10.00 AM) after an overnight fast. After 15 min in a seated position blood was drawn from an antecubital vein with a light tourniquet (40 mmHg). Blood was collected in citrated vacuum tubes and centrifuged for 20 min at 2000 $\times g$ after which platelet poor plasma and serum were stored at -80°C until analysis. Weight, waist- and hip circumference and blood pressure were measured in a standardized way. At the safety visit participants had a brief physical exam and routine blood work done. Adverse events were registered at every visit and participants were instructed to contact the investigators if they experienced any adverse effects.

Assays

Thrombin generation was assessed with a Calibrated Automated Thrombogram (Thrombinoscope BV, Maastricht, The Netherlands) using a fluorogenic method. After activation of the coagulation by adding tissue factor and phospholipids to platelet poor plasma five parameters were measured: Time to start of thrombin generation (lag-time, min); peak thrombin concentration (nmol/l); time to peak (min); area under curve (endogenous thrombin potential (ETP), nmol/l \times min) and start tail (min), i.e. the time where thrombin generation is terminated (35). Intra-assay CV 3.4–5.7% and inter-assay CV 2.9–9.0% for all parameters. Plasma levels of vWF-antigen were determined with a particle enhanced immunoturbidimetric assay, HemosIL von Willebrand Factor Antigen kit using an ACL 9000 System (Instrumentation Laboratory, Milan, Italy), plasma levels of PAI-1 were determined with an ELISA (antibodies: Mon-I-1 and Mon-I-6) on Tecan Sunrise plate reader (Tecan, Basle, Switzerland) and plasma levels of hsCRP using a CardioPhase hsCRP kit on a BNII protein analyzer (Siemens Healthcare Diagnostics GmbH, Marburg, Germany), with CV 4.6%, 6.4% and 3.1%, respectively. All analyses were performed at Unit for Thrombosis Research, Dept. of Clinical Biochemistry, Hospital of South West Denmark, Esbjerg, Denmark. Baseline and follow-up samples from each participant were ana

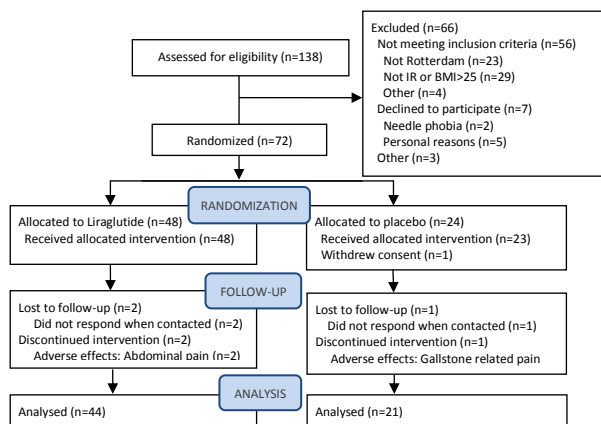


Figure 1. Participant flow

lyzed in the same batch. Plasma levels of insulin were determined using an electro-chemiluminescent immunoassay and a Cobas e411 reader (Roche Diagnostics GmbH, Mannheim, Germany), with intra-assay CV 2.1%. The Homeostasis Model Assessment of insulin resistance (HOMA2-IR) was calculated from fasting levels of insulin and glucose using an online HOMA-calculator (www.dtu.ox.ac.uk/homacalculator). Glucose, HbA1c, cholesterol and triglyceride levels were assessed using routine analyses at the Dept. of Clinical Biochemistry, Herlev Gentofte Hospital, DK. Androgen levels were determined using liquid chromatography and double mass spectrometry at Rigshospitalet, Copenhagen, DK.

Statistics

A sample size calculation based on an estimated standard deviation of 130 units obtained from in-house data, declared 63 subjects, randomized 2:1, needed for 80% power to find a difference in effect size of 100 nmol/min of ETP. This effect size was supported by a previous study finding a similar reduction in ETP with a 5% reduction in BMI (22). To allow for drop-outs 72 women were randomized. Distribution of data was checked using histograms and probability plots. Normally distributed data are presented as mean (SD), non-normally distributed data as median (p25-p75) and differences as mean (95%CI). Data were logarithmic transformed as appropriate, which is why some differences are presented as ratios. Fishers exact test was used for comparison of adverse effects between groups and paired t-test for quantification of effect (follow-up – baseline) in each group. In the initial protocol we planned on calculating the between-group difference using an unpaired t-test on the intention-to-treat population. Since a mixed model with maximum likelihood is a more optimal way of analyzing repeated measurements we have chosen this statistic approach and between-group differences in treatment effect are assessed using a repeated measurements mixed model (with maximum likelihood) with study drug as between-subjects effect and visit (time) as within-subject effect. Pre-specified subgroup analyses for Rotterdam phenotypes as well as four metabolic subgroups (HOMA2<2.3 + BMI<25; HOMA2<2.3 + BMI>25; HOMA2>2.3 + BMI<25 and HOMA2>2.3 + BMI>25) were performed using Mann Whitney U-test with Bonferroni correction. Associations between ETP and anthropometric, metabolic and endocrine parameters were assessed using uni-

Table 1. Baseline characteristics of the 72 PCOS women randomized

	LIRAGLUTIDE (N=48)	PLACEBO (N=24)	p
Age (years)	31.4 (24.6-35.6)	26.2 (24.8-31.5)	0.11
Weight (kg)	94.2 (15.4)	91.3 (13.6)	0.42
BMI (kg/m ²)	33.3 (5.1)	33.3 (4.6)	0.98
Waist (cm)	102.6 (10.8)	102.6 (11.1)	0.99
Waist/Hip ratio	0.91 (0.08)	0.92 (0.10)	0.75
Systolic / Diastolic BP (mmHg)	123 (9) / 79 (8)	124 (9) / 80 (7)	0.55 / 0.75
Smoking (<10 cigarettes/day)	18.8% (9)	33.3% (8)	0.24
First degree relative with DM2	31.3% (15)	26.1% (6) (n=23)	0.78
Ethnicity			0.68
Caucasian	93.8% (45)	91.7% (22)	
Rotterdam phenotype			0.54
O/A + HA + PCO	39.6% (19)	45.8% (11)	
O/A + HA	2.1% (1)	8.3% (2)	
HA + PCO	22.9% (11)	16.7% (4)	
O/A + PCO	35.4% (17)	29.2% (7)	
Ferriman Galloway score	7.0 (3.0-11.5)	7.0 (2.5-12.5)	0.90
Total testosterone (nmol/L)	1.23 (0.91-1.63)	1.35 (0.95-1.93)	0.46
SHBG (nmol/L)	31.0 (22.0-44.5)	30.5 (23.0-37.5)	0.68
Total cholesterol (mmol/L)	4.61 (0.80)	4.67 (0.57)	0.75
LDL Cholesterol (mmol/L)	2.83 (0.71)	2.99 (0.54)	0.30
HDL Cholesterol (mmol/L)	1.14 (0.25)	1.09 (0.28)	0.40
Triglycerides (mmol/L)	1.23 (0.90-1.63)	1.15 (0.90-1.47)	0.64
HbA1c (mmol/mol)	34.2 (2.8)	34.6 (3.4)	0.66
HOMA2-IR	2.29 (1.83-2.84)	2.42 (1.91-3.20)	0.25
GFR (ml/min/1.73 m ²)	112.8 (13.3)	117.5 (9.7)	0.12

Values are presented as mean (SD), median (p25-p75). Between-group differences at baseline were determined by un-paired t-test, Mann Whitney U-test, Chi²-test and Fisher exact test as appropriate.

and multivariate linear regression analyses on baseline data. In analyses regarding hsCRP estimates >10 mg/L were excluded. Statistical analyses were performed using SAS® Enterprise Guide 7.1 (SAS Institute Inc., U.S.). A two-sided p-value ≤0.05 was considered significant.

Results

Participant flow, baseline data and adverse effects

Of 138 women assessed for eligibility 72 were included of which 48 were randomized to liraglutide and 24 to placebo (Figure 1). Groups were comparable at baseline (Table 1). One woman in the placebo group withdrew her consent before starting treatment. Dropout ratio was 7/72 (9.7%) overall, 4/48 (8.3%) in the liraglutide and 3/24 (12.5%) in the placebo group. In the liraglutide group the most prevalent adverse effect was nausea, mainly in the up-titration phase (Table 2). Gallstone-related pain was experienced by three (6.4%) women in the liraglutide group and one (4.4%) in the placebo group and two women in the liraglutide group had a cholecystectomy. Due to discontinuation of study drug during diagnosis and surgery overall compliance for the two women was 64% and 87%, respectively. Self-reported median

Table 2. Adverse effects during 26 weeks liraglutide or placebo treatment

	Liraglutide (n=47)	Placebo (n=23)	p
Nausea	78.7 (37)	13.0 (3)	<0.01
Vomiting	10.6 (5)	0 (0)	0.2
Ructus/heartburn	17.0 (8)	0 (0)	0.05
Diarrhea	10.6 (5)	4.4 (1)	0.7
Constipation	25.5 (12)	0 (0)	<0.01
Gastroenteritis	10.6 (5)	8.7 (2)	1.0
Epigastric pain	17.0 (8)	0 (0)	0.05
Gallstone related pain	6.4 (3)	4.4 (1)	1.0
Cholecystectomy	4.3 (2)	0 (0)	1.0
Hypotension	2.1 (1)	0 (0)	1.0
Tachycardia	2.1 (1)	0 (0)	1.0
Syncope	2.1 (1)	0 (0)	1.0
Dizziness	8.5 (4)	0 (0)	0.3
Headache	0 (0)	13.0 (3)	0.03
Upper respiratory tract infection	14.9 (7)	17.4 (4)	1.0
Urinary tract infection	4.3 (2)	0 (0)	1.0
Hair loss	2.1 (1)	0 (0)	1.0
Rash at injection site	6.4 (3)	0 (0)	0.6
Joint pain	2.1 (1)	0 (0)	1.0

Values are presented as % (n). Adverse effects experienced at any point of the study period. Between-group differences were determined using Fishers exact test.

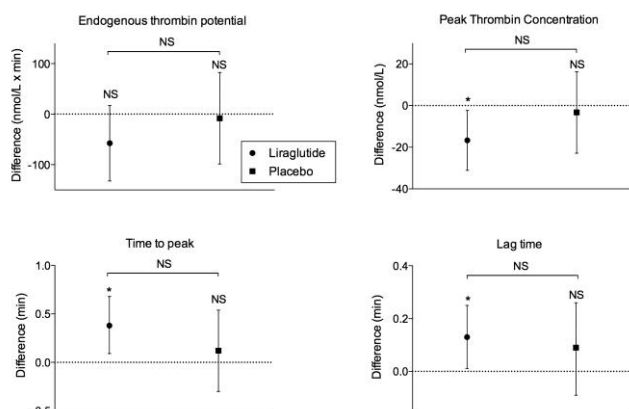


Figure 2. Change in thrombin generation parameters after 26 weeks of intervention. Dots and squares represent mean change (95%CI) from baseline to follow-up in the liraglutide and placebo group, respectively. Within-group and between-group comparisons are assessed with paired t-test and repeated measurements mixed model, respectively. * $p < 0.05$.

(p25-p75) compliance was 96% (89-99) in the active group and 93% (87-98) in the placebo group ($p = 0.70$). In the liraglutide group one (2%), 7 (15%) and 38 (83%) women completed the study at 0.6 mg/day, 1.2 mg/day and 1.8 mg/day, respectively. All women in the placebo group completed the study at the highest dose.

Anthropometric and metabolic measurements

Weight, BMI and waist circumference decreased significantly in the liraglutide group compared with the placebo group, -5.2 kg (95%CI 3.0-7.5, $p < 0.0001$), -1.8 kg/m² (95%CI 1.0-2.7, $p < 0.0001$) and -5.7 cm (95%CI 1.9-9.3, $p < 0.05$), respectively. When compared to placebo there was no effect of liraglutide on HOMA2-IR or on triglyceride and cholesterol levels (data not shown).

Thrombin generation, vWF, PAI-1 and hsCRP

In the liraglutide group peak thrombin concentration decreased by -16.71 nmol/L (95%CI -31.11- -2.32, $p < 0.05$) and lag-time and time to peak increased by 0.13 min (95%CI 0.01-0.25, $p < 0.05$) and 0.38 min (95%CI 0.09-0.68, $p < 0.05$), respectively (Figure 2). There was no effect on the primary outcome ETP (Table 3). Levels of PAI-1 decreased 12% (95%CI 0-23, $p = 0.05$) over six months in the liraglutide group and there was a trend towards decreased PAI-1 in the liraglutide group when compared with the placebo group ($p = 0.10$, Figure 3). We observed no differences between sub-groups with regard to effect on thrombin generation, vWF, PAI-1 or hsCRP levels.

Associations between ETP and anthropometric, metabolic and endocrine variables

At baseline ETP levels were associated with anthropometric and metabolic variables: BMI, waist circumference, diastolic blood pressure, HOMA2-IR, triglycerides, hsCRP and PAI1, and reciprocally associated with HDL and SHBG. Including BMI in the model, only levels of PAI-1 remained associated with ETP. Including HOMA2-IR, triglycerides and hsCRP in the model this association was no longer statistically significant.

Discussion

In this placebo-controlled randomized trial, investigating the effect of 26 weeks of liraglutide intervention on VTE and CVD risk markers in overweight women with PCOS we found a mean weight loss of 5.2 kg. Concomitantly we observed significant improvements in thrombin generation parameters: decreased peak thrombin concentration, increased lag time and time to

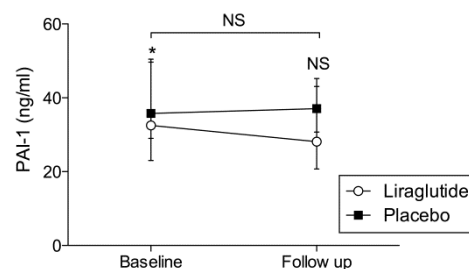


Figure 3. Levels of PAI-1 at baseline and 26-week follow-up. Within-group and between-group comparisons are assessed with paired t-test and repeated measurements mixed model, respectively. Data are presented as median (p25-p75) at baseline and follow-up. * $p = 0.05$.

peak. However, the changes observed disappeared when compared to placebo and appeared insufficient to impact the primary outcome, the total endogenous thrombin potential (ETP). However, in the liraglutide group we also demonstrated reduced PAI-1 levels and a trend towards a between-group difference, indicating improved fibrinolytic potential, and thus a net beneficial effect on validated markers of VTE and CVD risk.

To our knowledge, no previous studies have focused on the effect of liraglutide on this thrombin generation. An open-label, single-arm study investigating the effect of liraglutide (1.8 mg/day for six months) in 36 obese women with and without PCOS found improved endothelial function, seen as reduced levels of cell adhesion markers as well as slightly reduced clot lysis area, a complex measure of clot formation, density and lysis potential (29). Discrepancies might be explained by the single-armed design and their population having higher BMI and worse metabolic profile than ours. Moreover, clot formation is a measurement of platelet function and fibrin production, depending on thrombin concentration among numerous other factors (36), which is why our studies are not directly comparable.

Glintborg *et al.* recently studied the effect of metformin and combined oral contraceptives (COC) on thrombin generation in a RCT with 90 PCOS women (12). Thrombin generation, measured as ETP and peak thrombin concentration, increased after 12 months of intervention with COC, as well as with combined treatment (COC + metformin), but no changes were seen in the metformin alone group despite an almost significant median weight loss of 3 kg (37). The increase in thrombin generation was smaller in the combined group as compared with the COC group, suggesting a protective effect of metformin. Except from lower mean BMI their population was comparable to ours and the same thrombin generation assay was used.

Our hypothesis of weight loss and improved glycemic control resulting in reduced thrombin generation has been confirmed in other populations. Bariatric surgery in 36 morbidly obese adults (with and without type 2 diabetes) resulted in weight loss (mean -32%), reduced insulin resistance as well as decreased ETP and peak thrombin concentration at two-year follow-up (21). Additionally, one year of lifestyle intervention in 27 overweight children caused reduced BMI as well as decreased ETP and peak thrombin concentration (22). In a RCT Gram *et al.* found three months of daily endurance exercise to reduce BMI and ETP in 53 healthy, moderately overweight, young men, while there was no effect on peak thrombin concentration (23). Reasons for disagreement between our findings and the mentioned studies might be the excessive weight loss in the first (21), the lack of control group in the first and second (21,22) and possibly a "healthier" weight loss in the latter (23).

Table 3. Changes in pro-thrombotic and pro-inflammatory biomarkers from baseline to 26-week follow-up

	LIRAGLUTIDE			PLACEBO			DIFFERENCE BETWEEN GROUPS			
	Baseline (n=48)	Difference at six months (n=44)	p	Baseline (n=24)	Difference at six months (n=21)	p	Mixed models (crude)	p	Mixed models (adjusted) ^b	p
ETP (μmol/L x min)	1796 (332)	-57.6 (-132.3-17.2)	0.13	1830 (285)	-8.2 (-98.7-82.3)	0.85	-47.3 (-161.9-67.3)	0.41	-49.3 (-158.7-60.1)	0.37
Peak thrombin (nmol/L)	247.3 (41.1)	-16.7 (-31.1- -2.3)	0.02	245.7 (46.5)	-3.3 (-22.9-16.3)	0.73	-12.6 (-35.5-10.3)	0.27	-13.3 (-36.1-9.6)	0.25
Time to peak (min)	7.36 (1.11)	0.38 (0.09-0.68)	0.01	7.86 (1.19)	0.12 (-0.30-0.54)	0.56	0.25 (-0.25-0.75)	0.32	0.22 (-0.26-0.71)	0.36
Lag-time (min)	3.33 (2.92-3.67)	0.13 (0.01-0.25)	0.04	3.59 (3.00-3.84)	0.09 (-0.09-0.26)	0.32	0.04 (-0.17-0.24)	0.72	0.04 (-0.16-0.23)	0.71
Start tail (min)	26.00 (24.50-27.50)	0.42 (-0.34-1.18)	0.27	26.25 (25.00-28.75)	0.43 (-0.62-1.47)	0.40	0.99 (0.95-1.04) ^a	0.81	0.99 (0.95-1.04) ^a	0.79
vWF (% of normal)	99.5 (87.5-122.5)	1.64 (-3.3-6.6)	0.51	123.0 (92.0-145.0)	-5.2 (-12.1-1.7)	0.13	1.03 (0.96-1.11) ^a	0.35	0.91 (0.78-1.06) ^a	0.24
PAI-1 (ng/ml)	32.55 (23.05-49.65)	0.88 (0.77-1.00) ^a	0.05	35.75 (29.00-50.45)	1.04 (0.86-1.26) ^a	0.66	0.84 (0.68-1.04) ^a	0.10	0.83 (0.66-1.05) ^a	0.12
hsCRP (mg/L) ^c	2.05 (0.79-3.65)	0.85 (0.70-1.03) ^a	0.09	3.48 (1.17-4.32)	0.75 (0.43-1.30) ^a	0.29	1.02 (0.67-1.55) ^a	0.92	0.94 (0.64-1.39) ^a	0.76

Values are presented as mean (SD), median (p25-p75) and differences as mean (95%CI) or ^a ratio from logarithmic transformed numbers. ^b Mixed model are adjusted for age, BMI and smoking. ^c liraglutide n=45 (baseline), n=42 (follow-up), placebo n=20 (baseline), n=18 (follow-up).

Abbreviations: ETP = endogenous thrombin potential, PAI-1 = plasminogen activator inhibitor 1, hsCRP = high sensitivity C-reactive protein and vWF = von Willebrand factor.

Intervention with GLP-1 analogs has previously been shown to affect metabolic parameters in PCOS. In several smaller trials on women with PCOS GLP-1 analogs were found to reduce well-known risk factors of CVD: body weight, waist circumference and visceral adipose tissue (28,31,32), whereas results regarding HOMA-IR are conflicting (28–30) and results on hsCRP are sparse and inconsistent (28,29). In PCOS metformin seems to attenuate endothelial dysfunction and low-grade inflammation, evaluated as levels of PAI-1 and hsCRP (24,38–40); However, results are conflicting (41) and most of the studies are single-armed or open-labelled testing metformin vs. COC. Physical exercise was found to reduce BMI as well as levels of PAI-1 and hsCRP as compared to baseline in a six-month RCT on 136 women with PCOS (42). However, the effect disappeared when compared with placebo or COC (42). Also a lifestyle-induced weight loss of 8-11% was found to cause reduced levels of PAI-1 in an obese PCOS population (43). Since most interventional studies on endothelial function and low-grade inflammation in PCOS lack a placebo-arm, we cannot easily compare our findings.

Possibly GLP-1 analogs influence hemostasis in a directed way. The effect of GLP-1 analogs on hemostasis has been studied in vitro and in animal models. Human megakaryocytes seem to express GLP-1-receptors and human and murine blood incubated with the GLP-1 analog exenatide showed decreased in-vitro thrombus formation (44). Arterial thrombus formation decreased in mice treated with i.v. exenatide, suggesting GLP-1 analogs to reduce platelet aggregation by inhibiting the release of α- and dense granules (44). Moreover, liraglutide has been shown to attenuate high-glucose-mediated PAI-1 expression in human endothelial cells (45).

The anti-atherothrombotic potential of GLP-1 based therapies has been studied both in type 2 diabetes and obesity and have been found to reduce levels of hsCRP and PAI-1 in both conditions (26,27). In the recent LEADER trial including more than 9000 patients with type 2 diabetes and a concomitant cardiovascular condition liraglutide reduced the occurrence of CVD events and the rate of CVD death, as compared with placebo, as an add-on medication (46). The LEADER population was large and per-se at considerably greater risk of CVD events than our, younger popula-

tion still being relatively healthy with regard to having obtained a major cardiovascular burden.

This study has several strengths: the placebo-controlled RCT design, the high external validity and the low dropout rate. However, there are some limitations. An explanation for finding unaltered thrombin generation and levels of hsCRP and vWF with liraglutide therapy could be that our population was relatively “metabolically healthy”. Worth noting is the fasting glucose and lipid levels within normal range, despite the high mean BMI (33kg/m²) and waist circumference (103 cm). Inclusion of women being at a higher risk of CVD, i.e. with even higher BMI, reduced glucose tolerance, overt diabetes or dyslipidemia, might have given different results. This might also have been obtained by using the *National Institutes of Health* criteria instead of the Rotterdam criteria (3) and including older women. Our lack of findings might be due to lack of power. The fact that some of the TGT parameters were altered in the liraglutide group indicates that there might be an effect of the treatment, but the population might be too small to identify it.

In conclusion, in an overweight PCOS population 26 weeks of liraglutide intervention caused minor alterations in thrombin generation parameters, but no difference in overall thrombin generation when compared to placebo. We observed a substantial weight loss and a trend towards improved fibrinolytic potential. Taken together the results point towards beneficial effects on markers of VTE and CVD risk, which are promising, but need to be corroborated in larger studies. Liraglutide was well tolerated, although one should be aware of the risk of weight-loss-related gallbladder-stone-attacks in a population of young overweight women.

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Author contributions: JF, SOS and CK initiated the study and all five authors contributed to the protocol. SF and MN conducted the study and collected the data. All authors contributed to intellectual interpretation of the results. MN performed the statistical analyses and produced the manuscript, which all co-authors have read and approved.

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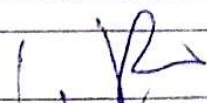
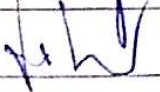
Title of PhD thesis:
Liraglutide in polycystic ovary syndrome: Effects on ovarian dysfunction and thrombin generation

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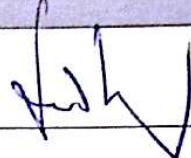
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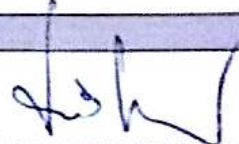
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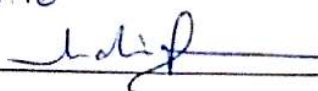
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