GLP-1R, a novel receptor in platelets; and the use of liraglutide in the treatment of obesity in women with PCOS

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Abstract:

Studies investigating atherothrombotic risk in women with polycystic ovary syndrome (PCOS), in particular platelet function and carotid intima-media wall thickness (cIMT), have been confounded by not adequately accounting for obesity. Liraglutide is a glucagon like peptide-1 (GLP-1) analogue that causes weight loss and may have favourable effects on atherothrombotic risk and liver fibrosis in preclinical and animal studies. The aims of this study was to investigate whether atherothrombotic risk was increased in obese women with PCOS independently of obesity; whether GLP-1 receptor (GLP-1R) is expressed in human platelets; and whether 6 months treatment with liraglutide would improve weight and markers of atherothrombosis and liver fibrosis in obese young women with PCOS and/or normal controls.

Our results suggest that PCOS, independent of obesity, is associated with increased levels of insulin resistance, inflammation, oxidative stress, non-alcoholic fatty liver disease (NAFLD) and the liver fibrosis marker PIIINP. However, PCOS was not independently associated with increased atherothrombotic risk markers including cIMT, platelet function, clot function/lysis, and endothelial function. Treatment for 6 months with liraglutide, 1.8mg daily, resulted in 3 – 4% weight loss in obese women with PCOS and controls. This was associated with a significant reduction in insulin resistance, oxidative stress, and several markers of atherothrombosis including inflammation, serum biochemical markers of endothelial function and clot lysis. Although basal platelet activation was reduced in the control group only and the liver fibrosis marker PIIINP was only reduced in the PCOS group, between groups comparisons were not significant. No change was observed in cIMT. In addition, we demonstrated for the first time that platelets express the GLP-1R. Liraglutide inhibited collagen- and thrombin-induced aggregation in isolated platelets and the effects were at least partly mediated by the GLP-1R, although an additional GLP-1R independent pathway is also likely.

In conclusion, cardiovascular risk in young obese women with PCOS can either be attributed to obesity or is not yet apparent at this early stage of the condition. Our data support the use of liraglutide as a weight loss medication in simple obesity and

suggest a potential beneficial effect on atherothrombotic risk and markers of liver fibrosis at 6 months of treatment. GLP-1R is a novel receptor in platelets and its function and clinical effect are worth further evaluation.

Publications:

Kahal H, Abouda G, Rigby A, Coady A, Kilpatrick E, Atkin S. Glucagon like peptide-1 analogue, liraglutide, improves liver fibrosis markers in obese women with polycystic ovary syndrome and non-alcoholic fatty liver disease. Clin Endocrinol (Oxf). 2013 Nov 21. doi: 10.1111/cen.12369. [Epub ahead of print]

Kahal H, Aburima A, Ungvari T, Rigby A, Dawson A, Coady A, Vince R, Ajjan R, Kilpatrick E, Naseem K, Atkin S. Polycystic ovary syndrome has no independent effect on vascular, inflammatory or thrombotic markers when matched for obesity. Clin Endocrinol (Oxf). 2013 Aug;79(2):252-8.

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Poster presentations:

International:

Kahal H, Kilpatrick ES, Coady AM, Atkin SL. The effects of treatment with liraglutide on quality of life and depression in young obese women with PCOS and controls. The International Society of Endocrinology and the Endocrine Society Meeting: ICE/ENDO 2014, Chicago, US, 21 – 24 June, 2014.

Kahal H, Abouda G, Rigby AS, Coady AM, Kilpatrick ES, Atkin SL. Glucagon like peptide-1 analogue, Liraglutide, improves liver fibrosis markers in obese women with polycystic ovary syndrome and non-alcoholic fatty liver disease. American Endocrine Society Meeting, San Francisco, US, 15 – 18 June, 2013.

Kahal H, Reid M, Coady AM, Kilpatrick ES, Atkin SL. Obesity explains the reduced quality of life found in polycystic ovary syndrome. American Endocrine Society Meeting, San Francisco, US, 15 – 18 June, 2013.

National:

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Kahal H, Aburima A, Ungvari T, Rigby A, Dawson A, Coady A, Vince R, Ajjan R, Kilpatrick E, Naseem K, Atkin S. Polycystic ovary syndrome has no independent effect on vascular, inflammatory or thrombotic markers when matched for obesity. British Endocrine Society Meeting, Harrogate, UK, 18 – 21 March, 2013.

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Contents

Abbreviations:

3βHSD	3β-hydroxysteroid dehydrogenase
8-iso $PGF_{2\alpha}$	8-iso prostaglandin F2α
17OHP	17α-hydroxyprogesterone
AC	Adenyl cyclase
ACD	Acid citrate dextrose
ACTH	Adrenocorticotropic hormone
ADP	Adenosine diphosphate
AF	Attachment Factor
ALT	Alanine aminotransferase
ANCOVA	Analysis of covariance
ApoE	Apolipoprotein E
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
AU	Arbitrary units
BMI	Body mass index
BP	Blood pressure
bpm	Beat per minute
BSA	Bovine serum albumin
CAH	Congenital adrenal hyperplasia
cAMP	Cyclic adenosine monophosphate
CCA	Common carotid artery
cDNA	Complementary DNA
CES-D	Centre for Epidemiologic Studies Depression Scale
cGMP	Cyclic guanosine monophosphate
cIMT	Carotid intima-media wall thickness
CV	Cardiovascular
DNA	Deoxyribonucleic acid
DPP-IV	Dipeptidyl peptidase IV
ECL	Chemiluminescence
ECM	Extracellular matrix

EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FAI	Free androgen index
FITC	Fluorescein isothiocyanate
FPG	Fasting plasma glucose
FSC	Forward scatter
FSH	Follicular stimulating hormone
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon like peptide-1
GLP-1R	Glucagon like peptide-1 receptor
GnRH	Gonadotropin releasing hormone
GPCR	Glycoprotein coupled receptor
HA	Hyaluronic acid
HbA1c	Glycosylated haemoglobin A1c
HDL	High density lipoprotein
HOMA-IR	Homeostasis model assessment-insulin resistance
HOMA-β	Homeostasis model assessment β-cell function
GP	Glycoprotein
HRP	Horseradish peroxidise
hsCRP	High sensitivity C-reactive protein
HUVECs	Human umbilical vein endothelial cells
ICC	Intraclass correlation coefficient
IDF	International Diabetes Federation
IFG	Impaired fasting glucose
IGR	Impaired glucose regulation
IGT	Impaired glucose tolerance
IP3	Inositol trisphosphate
IR	Insulin resistance
K_{ATP} channels	Adenosine-5’-triphosphate (ATP)-sensitive K^+ channels
LA	Lysis Area

LDL	Low-density lipoprotein cholesterol
LH	Luteinising hormone
LSGS	Low Serum Growth Supplement
LT	Lysis time
MA	Maximum absorbance
MDA	Malondialdehyde
MFI	Median fluorescence intensity
mRNA	Messenger Ribonucleic acid
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NC-CAH	Non-classic congenital adrenal hyperplasia
NIH	National Institute of Health
NO	Nitric oxide
OCP	Oral contraceptive pill
OD	Once a day
OGTT	Oral glucose tolerance test
PIIINP	Procollagen type III amino terminal peptide
PAI-1	Plasminogen activator inhibitor-1
PAT	Pulse wave amplitude
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCO	Polycystic ovaries
PCOS	Polycystic ovary syndrome
PCOSQ	PCOS health-related quality of life questionnaire
PDE	Phosphodiesterase
PE	Phycoerythrin
PGI_2	Prostacyclin
PGE_1	Prostaglandin E_1
PI-3K	Phosphatidylinositol 3-kinase
PKA	Protein Kinase A
PPP	Platelet poor plasma

PRP	Platelet rich plasma
PVDF	Polyvinyl-difluoride
QoL	Quality of life
REC	Research Ethics Committee
RHI	Reactive hyperaemic index
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase polymerase chain reaction
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SF-36	Short Form 36 Health Survey questionnaire
SHBG	Sex hormone binding globulin
sICAM-1	Soluble intercellular adhesion molecule-1
SSC	Side scatter
sVCAM-1	Soluble vascular cell adhesion molecule-1
T2DM	Type 2 diabetes mellitus
TBS-T	Tris base saline-tween
TEMED	N, N, N', N'-tetramethylenediamine
TSQM	Treatment Satisfaction Questionnaire for Medication
TV USS	Trans-vaginal ultrasound
TxA2	Thromboxane A_2
VASP	Vasodilator stimulated phosphoprotein
vWF	Von Willebrand factor
WB	Western blotting
WBC	White blood cells
WHOQOL-BREF	World Health Organization QoL questionnaire
WHR	Waist-to-hip ratio

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Author's declaration

I confirm that this work is original and that if any passage(s) or diagram(s) have been copied from academic papers, books, the internet or any other sources these are clearly identified by the use of quotation marks and the reference(s) is fully cited. I certify that, other than where indicated, this is my own work and does not breach the regulations of HYMS, the University of Hull or the University of York regarding plagiarism or academic conduct in examinations. I have read the HYMS Code of Practice on Academic Misconduct, and state that this piece of work is my own and does not contain any unacknowledged work from any other sources. I confirm that any patient information obtained to produce this piece of work has been appropriately anonymised.

Author's contribution:

My personal contribution to the work presented here includes:

- Contributing to the research questions and design of studies performed.
- Writing the clinical trial protocol and patient information sheet, and applying for Ethics and Medicines and Healthcare Products Regulatory Agency (MHRA) approvals.
- Recruiting study participants and collecting clinical data including anthropometric measurements, blood samples, and quality of life measurements.
- Performing all EndoPAT-2000, cIMT, and platelet function studies on clinical study participants and measuring urinary isoprostane.
- Performing laboratory experiments including: platelet isolation, platelet aggregation, flow cytometry, phospho-flow, western blotting, reverse transcription polymerase chain reaction (RT-PCR), cell culture, and enzyme-linked immunosorbent assay (ELISA).
- Analysing data, and writing the thesis.
- Presenting data at conferences and submitting work to peer-reviewed journals.

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1. Chapter 1: Introduction

1.1 Polycystic Ovary Syndrome.

Polycystic ovary syndrome (PCOS) is a syndrome of ovarian dysfunction. It is a common disorder with a prevalence of 6 – 15% of women of reproductive age (1-5). The clinical picture commonly includes obesity, hirsutism, oligmenorrhoea, and subfertility.

1.1.1 Definition.

In 1990, an expert panel at the National Institutes of Child Health and Human Development conference recommended that chronic anovulation with biochemical and/or clinical hyperandrogensim are essential for making the diagnosis of PCOS, this is commonly known as the NIH 1990 criteria (6). Women who fulfil these criteria are thought to have 'classic PCOS'.

In 2003, the European Society for Human Reproduction and Embryology and the American Society for Reproductive Medicine co-sponsored the Rotterdam polycystic ovary syndrome consensus workshop that revised and broadened the NIH 1990 diagnostic criteria (7). The revised Rotterdam criteria considered the presence of polycystic ovaries on ultrasound (defined as either 12 or more follicles measuring 2–9 mm in diameter, or an ovarian volume of >10 cm^3) of equal diagnostic importance to anovulation and hyperandrogenism (7). Under these criteria, any two of the three components are sufficient for making the diagnosis of PCOS. This created a total of four groups of women with PCOS; hyperandrogenism and oligomenorrhoea with or without polycystic ovaries on ultrasound, 'classic PCOS'; and hyperandrogenism but regular menses or those with menstrual disturbance without overt androgen excess, 'non-classic PCOS'.

In 2006, the Androgen Excess Society recommended that hyperandrogenism should be considered an essential component of PCOS (8). The panel recommended the combination of biochemical or clinical hyperandrogenism with chronic anovulation or polycystic ovaries on ultrasound for making the diagnosis of PCOS.

The three diagnostic criteria for PCOS are summarised in Table 1.1. Although the revised Rotterdam criteria are commonly used, they are still controversial, especially as the isolated finding of polycystic ovaries, which meets the classic

ultrasonographic definition, occurs in 16–25% of the normal population (9). This controversy surrounding the diagnostic criteria for PCOS reflects the heterogeneous presentation of the syndrome and the advances in understanding its uncertain aetiology (10).

NIH (6)	Hyperandrogenism and oligomenorrhoea /anovulation
Rotterdam (7)	Two of the following: hyperandrogenism, oligomenorrhoea /anovulation, and polycystic ovaries on ultrasound
Androgen Excess Society (8)	Hyperandrogenism plus oligomenorrhoea/anovulation and/or polycystic ovaries on ultrasound

Table 1.1 Diagnostic criteria for PCOS. All criteria require the exclusion of other disorders: non-classic congenital adrenal hyperplasia (NC-CAH), androgen secreting tumours, and Cushing's syndrome must be excluded in women with raised androgens; thyroid disorders and raised prolactin should be excluded in women with menstrual disturbances. Oligomenorrhoea is defined as mean cycle length > 35 days (usually fewer than eight cycles per year) (11, 12).

1.1.2 Aetiology.

As yet, we do not understand the aetiology of PCOS, although both genetic and environmental influences are implicated (13, 14). A few theories have been proposed:

1.1.2.1 Genetic susceptibility:

PCOS is likely to be a complex genetic disease with at least several susceptibility genes (15). In support of this theory is the high prevalence of PCOS in some families (16), and in twin studies (17). Factors that may contribute to the phenotypic variation within families may include obesity, insulin resistance, additional genes and environmental factors (12).

Candidate gene association studies attempting to identify causative loci for PCOS in genes involved in androgen biosynthesis and secretion, gonadotrophin secretion, the secretion and action of insulin and folliculogenesis have been largely

disappointing and limited by small samples size and imperfect matched controls (12, 15). A meta-analysis of small case-control studies suggested an association between single nucleotide polymorphisms (SNPs) in the insulin receptor substrate-1 (IRS-1) and PCOS (18).

Recently, genome-wide association studies (GWAS) have identified one locus on chromosome 9, linked to the DENND1A gene, in which the minor allele confers an increased risk of PCOS in both Han Chinese, Caucasian and Icelandic populations (19-21). Other loci linked to PCOS in Han populations (19), THADA and LHCGR on chromosome 2, are not found consistently in European populations (22). While LHCGR gene encodes the receptor for luteinising hormone (LH) and human chorionic gonadotropin and subsequently is a plausible PCOS candidate (12), questions arise as to the pathophysiological links of the two other loci (THADA and DENND1A) to reproduction or ovarian function (22). A study by Welt et al. (21) found a significant association between one variant in DENND1A and testosterone levels, supporting the hypothesis that hyperandrogenaemia was the trait most likely to have a genetic basis in PCOS (23). While another variant in DENND1A was significantly associated with hyperandrogenism and irregular menses but not with polycystic ovarian morphology (21), which suggests that there is genetic susceptibility for PCOS diagnosed by the NIH rather than the Rotterdam criteria (12).

1.1.2.2 Steroidogenic defect.

An increase in circulating androgens of ovarian origin is one of the main features of PCOS and is present in both ovulatory and anovulatory women. Studies investigating whether increased circulating androgens were due to increased follicle number or an intrinsic defect of these follicles (24) demonstrated that androstenedione accumulation was approximately 20-fold higher per cell in the theca from women with PCOS than in normal controls suggesting an intrinsic abnormality of theca cell function. Interestingly, androgen production was increased whether the theca originated from ovulatory or anovulatory polycystic ovaries (25). In addition, production of 17α-hydroxyprogesterone (17OHP) and progesterone were also increased, suggesting increased activity of P450 17α

hydroxylase; 17, 20 lyase and 3β-hydroxysteroid dehydrogenase (3βHSD) (Figure 1.1). Subsequently, theca cells from polycystic ovaries secrete more androgens, basally and in response to stimulation (Figure 1.2) (24).

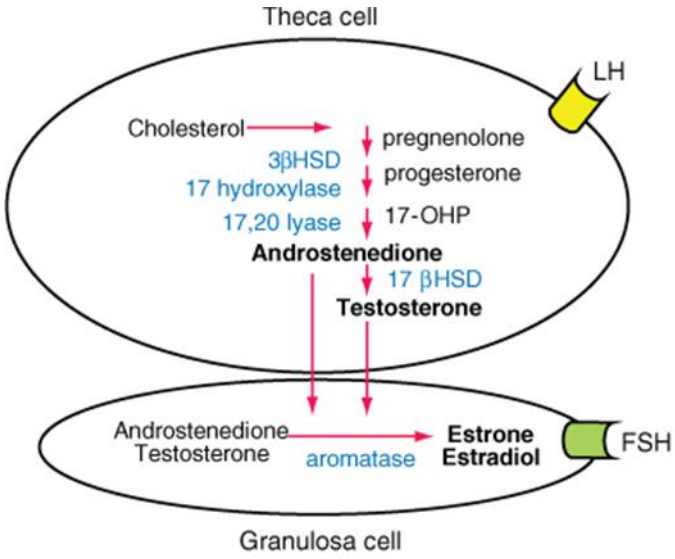


Figure 1.1 Ovarian steroidogenesis. Estrogen production in the ovary requires the cooperative function of the theca and granulosa cells under the control of luteinising hormone (LH) and follicular stimulating hormone (FSH). 3βHSD, 3β-hydroxysteroid dehydrogenase; 17-OHP, 17α-hydroxyprogesterone; Estrone, oestrone; Estradiol, oestradiol. This figure was obtained from Fauci et al. Harrison's principles of Internal Medicine (26).

In both ovulatory and anovulatory polycystic ovaries (PCO), the proportion of early growing (primary) follicles is significantly increased compared with normal ovaries (27). Animal studies suggest that prenatal exposure to androgens may stimulate the initiation of follicular development and produce phenotypic features of PCOS in the female offspring, as suggested by studies in pregnant Rhesus monkeys and sheep (28, 29). However, this may not reflect what happens in humans as although maternal androgen levels are elevated in PCOS pregnancies (30), it is unlikely that they reach the fetus because of placental aromatase activity (31, 32). The excess of follicles could result from accelerated follicle growth and/or prolonged survival of small follicles in comparison to follicles from normal ovaries (27, 33). While granulosa cells from ovulatory women with PCO are similar in terms of responses to

follicular stimulating hormone (FSH) and oestradiol production to those from normal women (34, 35), granulosa cells isolated from antral follicles obtained from anovulatory women with PCO showed increased oestradiol production in response to FSH and premature responsiveness to LH (34, 35). These abnormalities may contribute to the arrest of follicular development in PCO, while in normal ovaries the lack of follicular development and ovulation may be secondary to lower levels of circulating FSH (12, 36, 37).

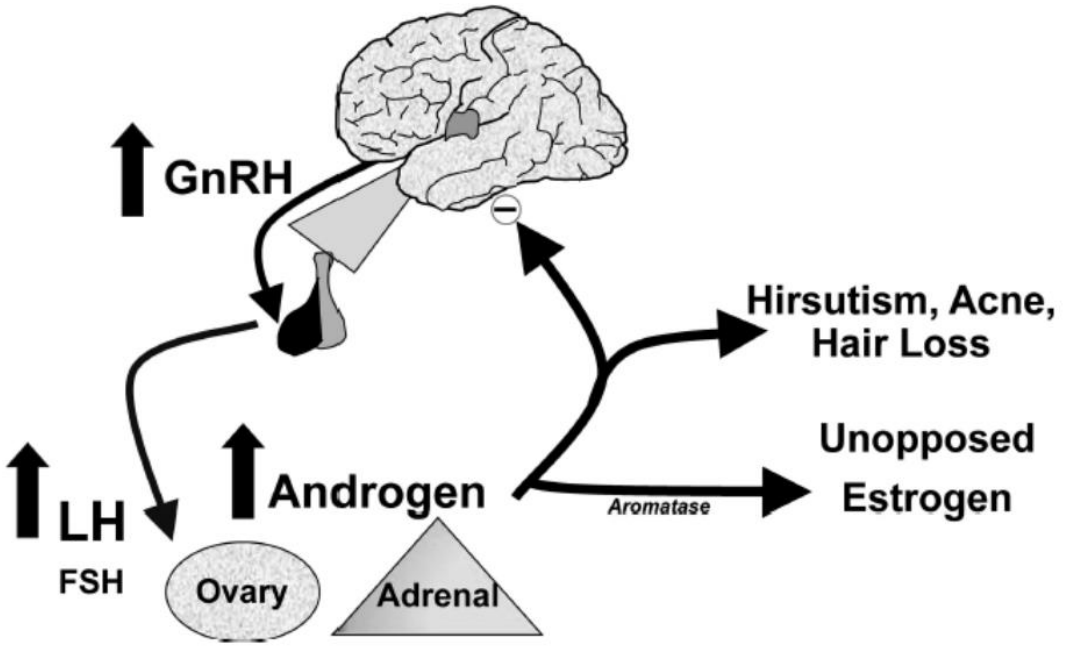


Figure 1.2 The role of androgens in the pathophysiology of PCOS. There is increased frequency of pulsatile GnRH release that selectively increases LH secretion. LH stimulates ovarian theca cell testosterone production. Testosterone is incompletely aromatized by the adjacent granulosa cells because of relative FSH deficiency. There are also constitutive increases in the activity of multiple steroidogenic enzymes in polycystic ovaries contributing to increased androgen production. Increased adrenal androgen production may also be present in PCOS. Testosterone acts in the periphery to produce signs of androgen excess, such as hirsutism, acne, and alopecia. Testosterone and androstenedione can also be aromatised extragonadally to oestradiol and oestrone, respectively, resulting in unopposed oestrogen action on the endometrium. Testosterone feeds back on the hypothalamus to decrease the sensitivity to the normal feedback effects of

oestradiol and progesterone to slow GnRH pulse frequency. LH, luteinising hormone; GnRH, Gonadotropin releasing hormone; FSH, follicular stimulating hormone; Estrogen, oestrogen. This figure was obtained from Diamanti-Kandarakis E and Dunaif A, 2012 (12).

1.1.2.3 Insulin resistance.

More than 50% of women with PCOS, independent of obesity, are insulin resistant (IR) (38). The glucose uptake pathway is resistant in muscle and adipose (39) and has also been demonstrated to be abnormal in granulosa cells (40). Conversely, the ability of insulin to promote ovarian steroidogenesis does not seem to be impaired. Insulin may act directly on the ovaries to enhance androgen production through the insulin receptor, insulin growth factor-1 receptor and/or by potentiating the LH effects on the ovaries (12). Insulin may also lead to hyperandrogensim indirectly by reducing sex hormone binding globulin (SHBG) production from the liver with subsequent increase in bioavailable testosterone. Insulin has also been implicated in causing the premature arrest of follicle growth (34, 41). In support of this theory an extensive body of literature indicating that lowering insulin levels with insulin-sensitizing drugs, metformin (42) and the thiazolidinediones (43), can reduce circulating androgen levels, increase SHBG levels, and restore ovulatory menstrual cycles in women with PCOS (12).

Women with chronic anovulation and hyperandrogenism, with or without ultrasound features of PCOS 'classic PCOS' appear to be at substantially greater risk for IR than those with hyperandrogenism and regular cycles 'non-classic PCOS' (44, 45).

One of the difficulties that faces any theory trying to explain the origin of PCOS is that it is not one entity. Women with PCOS are not all IR, and the presence of hyperandrogenism and oligo/amenorrhoea are not necessary for making the diagnosis. However, the ability to move between ovulatory and anovulatory cycles as a result of dietary factors or advancing reproductive age (women with PCOS tend to have more regular cycles as they enter their late thirties and forties) (46) provides persuasive evidence that PCOS represents a wide, but single, spectrum of

disordered ovarian and metabolic function. One condition which combines both mechanisms, IR and hyperandrogenism, is obesity, as discussed below.

1.1.3 Clinical Presentation.

PCOS commonly presents during the teenage years and symptoms tend to improve as women get older (47). Women with PCOS commonly attend the clinic with one or more of these complaints; hirsutism, menstrual irregularity, subfertility, and obesity. In the next few paragraphs I will give a brief review of each of these symptoms with special focus on obesity.

1.1.3.1 Hirsutism.

Hirsutism is the commonest presentation in PCOS; hirsutism and/or hyperandrogenism are considered by many authors essential elements in making the diagnosis of PCOS (48, 49). However, hirsutism is subjective and many women have unrealistic expectations about what they consider to be 'normal' body hair distribution.

The hair growth cycle includes three phases; anagen, the active phase of hair growth; telogen, a period when the hair is no longer growing; and catagen, a period when the hair is pushed away from the follicle and shed (50). Vellus hair is nonmedullated, short, soft, and lightly pigmented. Terminal hair is medullated, longer, stiff, and pigmented. In contrast to vellus hair, terminal hair is influenced by androgen levels. Hirsutism in women develops when terminal hair is present in areas including shoulders, back, chest (apart from a few periareolar hairs), upper abdomen and face (apart from few hairs on upper lip and chin) (50). Terminal hair in other areas is considered normal. While clinical scores to assess hirsutism could be helpful, for example the Ferriman-Gallwey score (51), their clinical use is limited by the fact that many women remove any extra hair before attending the clinic.

Hirsutism in PCOS usually starts during the teenage years and progresses slowly. Biochemical screening may be normal but commonly shows elevated levels of testosterone, and/or free androgen index, and/or androstenedione (7). As the levels of these hormones change during the menstrual cycle they should ideally be

checked at 9am at the follicular phase of the cycle, which might be difficult to achieve in the presence oligo/amenorrhoea.

It is important to rule out other less common causes of hirsutism including androgen producing tumours, Cushing's disease, and NC-CAH (52, 53). Hirsutism due to androgen producing tumours commonly presents later in life and progresses rapidly. It may also be associated with masculinisation, for example temporal balding and hoarse voice; or virilisation, for example clitoromegaly. Testosterone levels are usually much higher than those seen in PCOS; more than two times above the upper limit of normal (53).

NC-CAH is usually caused by partial deficiency in the enzyme 21 hydroxylase in the adrenal cortex, inherited in an autosomal recessive manner, and it results in over production of androgens from the adrenals. The diagnosis is made by confirming elevated levels of 17OHP in response to stimulation with adrenocorticotropic hormone (ACTH) (short synacthen test). Although some authors do not routinely test for NC-CAH, confirming the diagnosis is important as patients may benefit from genetic counselling before starting a family and may need steroid replacement to improve hirsutism and achieve fertility (54).

Treatment of hirsutism is difficult as the effects of most treatments are mild to moderate and patients' expectations are sometimes unrealistic. Mechanical treatments including shaving, plucking and waxing are essential as all other treatments require a few months to reach maximum effect. Local treatments include eflornithine cream which shortens the anagen phase of hair growth (55); electrolysis, in which an electric current is applied to a fine wire and inserted into a hair follicle at the anagen phase to destroy it; however, it is expensive, painful, time consuming and operator dependent (50); and laser therapy, in which selective heating of hair melanin with subsequent thermal injury to the follicle. Though effective it is dependent on skin and hair colour, expensive, and painful (56). Oral agents include the combined oral contraceptive pill (OCP), with a progesterone component that has an anti-androgen effect, for example cyproterone acetate or norgestimate (however, there is increased risk if thromboembolic events and breast cancer); spironolactone, androgen receptor blocker (57); flutamide, androgen

receptor blocker (use is limited by increased of risk of hepatic toxicity) (58, 59); and finasteride, inhibit type II 5-α reductase and blocks the conversion of testosterone to the more potent androgen dihydrotestosterone. All oral treatments are contraindicated during pregnancy.

1.1.3.2 Menstrual irregularity.

Oligomenorrhoea (less than 9 periods a year) and amenorrhoea (cessation of menses for > 6 months) are common presentations in women with PCOS (7). Assessing period regularity during teenage years is often difficult as periods are commonly irregular in normal girls during the first two years after menarche (5). Other conditions which may present with oligo/amenorrhoea include hyperprolactinaemia, hypogonadotropic hypogonadism, premature ovarian failure, thyroid abnormalities, and simple obesity. Biochemical screening in PCOS commonly reveals raised LH and normal oestrogen levels (7).

Periods' regularity tends to improve as women with PCOS get older (47). Lifestyle changes are considered first line treatment in PCOS (60). While weight loss has been found to improve periods' regularity, the impact of exercise alone is less obvious (61). Metformin is a biguanide that reduces plasma insulin levels and may improve periods' regularity in some women with PCOS (62-64). Although the OCP regulates periods, its use might be limited in obesity because of increased risk of thromboembolic disease. It has been suggested that amenorrhoeic women with PCOS should have at least four periods a year to reduce the risk of endometrial hyperplasia (65). This could be achieved with a short course of progesterone, for example medroxyprogesterone 10mg bd for 10 days, every three months.

1.1.3.3 Fertility.

Women with PCOS are more likely to suffer from anovulation and subfertility (61). Obesity is also associated with subfertility (66) and several prospective studies in obese women with and without PCOS suggest improved fertility after 5% or more weight loss (67-69). However, although lifestyle changes are recommended (61), there is a lack of randomised controlled trials comparing the effects of lifestyle intervention to minimal or no treatment on fertility (pregnancy, live birth and

miscarriage) in women with PCOS (60). Clomiphene citrate is first line treatment to improve ovulation and fertility in women with PCOS (61, 70). Women who do not respond to clomiphene citrate may benefit from assisted fertility (61). Metformin was not found to improve fertility (71, 72), though there might be a place for metformin in the treatment of women with PCOS with clomiphene-resistant infertility (73).

1.1.3.4 Obesity.

Around 30 – 88% of women with PCOS are overweight or obese (1, 74-76); this figure varies somewhat depending on ethnicity and geography. While weight gain is associated with an increasing chance of chronic anovulation; diet and lifestyle changes in obese women with PCOS have beneficial effects on many clinical features of PCOS including ovulation (77).

A. The role of obesity in the development of PCOS.

The exact role that obesity plays in the development of PCOS remains to be determined. Obese and lean women with PCOS are believed to have increased android type adiposity, adipose tissue distributed mainly in visceral and abdominal subcutaneous depots, compared to controls (78, 79). Android type adiposity carries an increased risk of IR. The adipose tissue expandability theory (80) proposes that at a set point of positive energy imbalance, which is determined on an individual basis, by genetics and environmental factors, the subcutaneous adipose tissue fails to expand to store more fat subcutaneously. This results in a state of lipotoxicity and fat starts to deposit in other tissues including the liver, muscles and pancreas. Lipotoxicity drives IR and subsequently hyperandrogenism commonly seen in women with PCOS (81). Obesity may also play a direct role in the development of hyperandrogenism through altered steroid metabolism in adipocytes. The conversion of 'weaker' androgens to more potent ones takes place in peripheral tissues, including adipocytes. Increased 17βHSD activity has been noted in simple obesity with subsequent increased conversion of androstenedione to testosterone. Increased activity of the enzyme 5α-reductase, which converts testosterone to the more potent dihydrotestosterone, has also been found in women with PCOS (82).

Although, altered adipokine levels have been suggested in the development of IR and PCOS (83, 84), the data have not been consistent.

It is therefore plausible to propose that obesity plays a crucial role in the development and presentation of PCOS. It is possible that many obese women with PCOS may have remained asymptomatic, had they not become obese (76). It is no surprise that tackling obesity is considered by many clinicians as the first step in managing women with PCOS.

B. Treatment of obesity in the setting of PCOS.

Even modest weight loss of 5 – 10% of initial body weight has been shown to increase the frequency of ovulation, improve conception, and reduce testosterone, free androgen index (FAI), hyperlipidaemia, hyperglycaemia, and IR in women with PCOS (85-88).

I. Life style changes.

Lifestyle modification is regarded as the first line treatment for women with PCOS. Exercise and weight loss improve insulin sensitivity with 44 – 57% of women with PCOS reporting improvement in either menstrual cycle or ovulation after lifestyle changes and subsequent weight loss (85, 88, 89). Although different diets have been proposed, there is no strong evidence that one diet is superior to another in inducing weight loss in PCOS. Hence, weight loss is probably more important than the dietary composition in improving symptoms of PCOS (90).

One of the main challenges of lifestyle modification is low participants' compliance rate over time (91). In a systematic review and meta-analysis of studies investigating the effectiveness of dietary interventions +/- exercise in long-term weight loss, almost half the initial weight loss achieved was regained at one year in both groups (91). Therefore, pharmaceutical intervention is an additional essential therapeutic tool to lifestyle changes in many patients.

II. Medical therapy.

Only a few safe and effective drugs are currently available for the treatment of obesity. Although sibutramine (92) and rimonabant (93) have been shown to be

effective in inducing weight loss in PCOS women, they have both been withdrawn from the UK. Rimonabant increased the risk of psychiatric disorders (94) while sibutramine has been associated with hypertension and cardiovascular (CV) disease (95).

a) Orlistat.

Orlistat is a lipase inhibitor that reduces fat absorption in the gut by approximately 30% (96). In a meta-analysis it was estimated that orlistat treatment led to an average placebo-subtracted weight loss of 2.7 kg at 1 year (97). In a randomised controlled trial of 3305 obese subjects, orlistat treatment was associated with a 3.6 kg weight loss compared with 1.4 kg for placebo at 4 years (98). Studies using orlistat in women with PCOS were of shorter duration and included a smaller number of participants. Three months treatment with orlistat in obese women with PCOS were associated with a 4.69% weight reduction and improvement in total testosterone levels (99) and reduction in IR (100).

The use of orlistat is limited by its gastrointestinal side effects. Approximately 15 - 30% of those taking orlistat experience oily stool, faecal urgency, or oily spotting, and 7% report faecal incontinence, particularly at the initiation of treatment (97). Despite its relatively high rate of side effects, orlistat is a useful treatment tool in the management of obese women with PCOS.

b) Metformin.

Metformin is a biguanide commonly used for the treatment of type 2 diabetes (T2DM). Metformin's primary action is on the liver, in which it reduces gluconeogenesis. Extrahepatic sites of action include the skeletal muscles, adipose tissue, endothelium, and the ovary (101, 102). Metformin is commonly used in women with PCOS and is reported to improve IR, SHBG, hyperandrogenism and ovulation (63, 64). Metformin's effect on weight management is probably negligible. In a systematic review and meta-analysis [54] metformin was found to have no effect on body weight and body mass index (BMI) in women with PCOS (103). Interestingly, metformin when given to obese PCOS women after rimonabant maintained the weight reduction and decrease in waist circumference achieved by

rimonabant and augmented the initial improvements in testosterone levels and IR (104). The authors suggested that the insulin sensitisation action of metformin was possibly complementary to the weight loss caused by rimonabant (104).

Despite the lack of evidence of a beneficial effect for metformin on body weight it remains to be widely used in PCOS probably for its effect on IR. The most frequent side effects of metformin are gastrointestinal symptoms including nausea, anorexia, vomiting, abdominal discomfort, and diarrhoea, and occur in up to 20% of patients. In 3 -5% of patients, therapy may have to be discontinued because of these adverse effects (102, 105). A slow release form of metformin is available and is believed to be better tolerated (106).

c) Incretin mimetic therapy.

The gastrointestinal tract produces several peptide hormones that participate in regulation of food intake. Ingested nutrients, especially fats and carbohydrates, stimulate glucagon like peptide-1 (GLP-1) secretion from L cells in the distal small intestine (107). GLP-1 accentuates glucose-dependent insulin release, inhibits glucagon secretion, increases pancreatic β cell growth, suppresses appetite and energy intake and delays gastric emptying (108-111). Glucagon-like peptide-1 receptor (GLP-1R) is expressed in the gut, pancreas, brainstem, hypothalamus, and vagal-afferent nerves (108). Activation of the hypothalamic GLP-1R decreases food intake (112).

In a 24-week randomised controlled trial in women with PCOS (113) a combination treatment with exenatide (GLP1 mimetic), and metformin was found to be superior to exenatide or metformin monotherapy in reducing weight (mean weight loss of 6 ± 0.5 kg) and improving menstrual cycles, ovulation rate, FAI, and insulin sensitivity. In a 20-week randomised open labelled study involving obese healthy men and women (114), comparing liraglutide (GLP1 analogue) to orlistat, it was shown that liraglutide treatment was well tolerated, and induced significant dose-related weight loss (mean weight loss 4.8 – 7.2 kg) compared to placebo or orlistat. All the patients were obese and had a 500 kcal per day energy deficit diet and increased their physical activity throughout the study. In a head-to-head comparison study in

people with T2DM, 1.8 mg liraglutide daily and 10µg exenatide twice daily produced similar weight loss (3.2 kg with liraglutide vs. 2.9 kg with exenatide) (115). However, liraglutide achieved better glycaemic control and was better tolerated than exenatide (115).

Most commonly reported side effects are nausea and vomiting, but the main safety concern remains a possible increased risk of pancreatitis attributable to drugs that act through the GLP-1 pathway (116). One of the disadvantages of using GLP-1R agonists is that they require injection. Although, not yet licensed for obesity management, GLP-1R agonists offer a potential obesity treatment for women with PCOS. In the next few chapters I will be examining the use of GLP-1 analogue, liraglutide, in the treatment of obesity in women with PCOS.

III. Other treatments.

Other treatments for obesity including behavioural therapy and bariatric surgery have also been found to be effective in reducing weight and improving symptoms of PCOS (117-119). Bariatric surgery was associated with a significant improvement in weight, hirsutism, IR and fertility in women with PCOS (117, 118). It has also been suggested, in a recent Australian evidence-based guidelines, that bariatric surgery should be considered and initiated at a lower cut-off (BMI ≥35 kg/m^2) in infertile women with PCOS who are anovulatory and remain infertile after intensive multidisciplinary lifestyle management for a minimum of 6 months (120, 121).

1.1.4 Cardiovascular risk in PCOS.

Many CV risk factors have been found to be increased in PCOS including; impaired glucose tolerance (IGT) and T2DM (122), dyslipidaemia (123), endothelial dysfunction (124), platelet dysfunction (125), and increased carotid intima-media wall thickness (cIMT) (126). However, obesity is an important confounder which many studies have failed to account for when assessing CV risk in PCOS.

Using a risk assessment model Dahlgren et al. (127) predicted that PCOS carries a seven fold increase in relative risk for myocardial infarction. This assumption was disputed by Wild et al. (128) who performed a retrospective cohort study of 319 women with PCOS, average follow up of 31 years, and 1060 age matched controls.

The authors found no difference between the two groups in all-cause mortality, cardiovascular mortality or cardiovascular events, despite the PCOS group showing higher prevalence of diabetes, hypertension and hyperlipidaemia, after adjustment for BMI (128). Only non-fatal cerebrovascular events were found to be higher in the PCOS group (128). In another study, Shaw et al. (129) examined 104 postmenopausal women with PCOS and 284 controls. PCOS was diagnosed retrospectively depending on self-reported premenopausal history of oligomenorrhoea and postmenopausal presence of elevated androgens. All women underwent coronary angiography for suspected ischaemic heart disease and were followed up prospectively. The authors found the 5 year CV event-free survival (CV death or nonfatal MI) to be lower for women with PCOS compared to controls, 78.9% vs. 88.7%, respectively (P=0.006) (129). However, the study is limited as the diagnosis of PCOS was made retrospectively, only 20% had previous diagnosis of PCOS, and the hyperandrogenaemia which was identified postmenopausally may not necessarily be present when the women were premenopausal. In a recent retrospective cohort study, Mani et al. (130) reviewed CV events in a group of 2301 women with PCOS using hospital electronic records. Mean age at the start of the observation was 29.6 ±10 years and at the end of observation was 36.3 ±10 years. The study showed an increased prevalence of angina and myocardial infarction in the PCOS group compared to an age-matched local female population, with odds ratios ranging from 2.6 – 12. 9 with the highest ratio being for myocardial infarction in the group >65 years old (130). However, the study is limited by its retrospective design, lack of a control group, missing data including how the diagnosis of PCOS was made in 27% of participants, and the fact that women with PCOS were identified from a tertiary centre clinic and may not necessarily reflect women with PCOS in the community. Nevertheless, it is still arguable if PCOS independently carries increased CV morbidity and mortality. The heterogeneous presentation of the condition and the young age of diagnosis made it difficult to answer this question. We are still awaiting prospective, large, well conducted trials in PCOS, with long duration of follow up to assess CV outcome. Till then we are left with

assumptions through examining surrogate markers for CV risk in PCOS. In this chapter I will be focusing on two of these markers; cIMT and platelet function.

1.2 Carotid intima-media wall thickness.

Decades of silent arterial wall alterations precede vascular clinical events, which reflect advanced atherosclerotic disease. The first morphological abnormalities of arterial walls can be visualized by B-mode (brightness mode) ultrasonography (Figure 1.3). This high-resolution, non-invasive technique is one of the best methods for the detection of early stages of atherosclerotic disease, because it demonstrates the wall structure with better resolution than other similar techniques, e.g. magnetic resonance imaging (131).

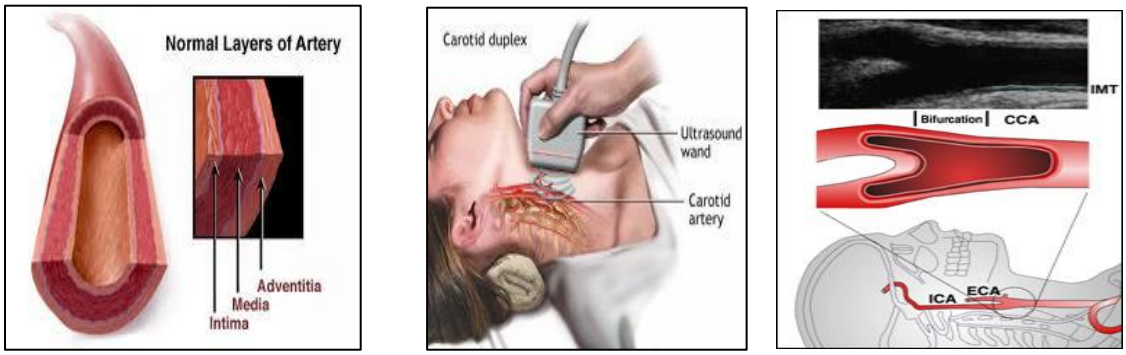


Figure 1.3 Demonstration of normal layers of artery and cIMT measurement. The intima is the innermost layer of the artery, and the media is the middle layer of the artery. IMT: intima-media thickness, CCA: common carotid artery, ECA: external carotid artery, ICA: internal carotid artery (132-134).

1.2.1 cIMT and cardiovascular risk.

cIMT was first described in 1986 (135) and was found to correlate well with artery wall histology (135). An increased cIMT is associated with CV risk factors such as smoking, hypertension, cholesterol levels, diabetes, and age (Figure 1.4) (131, 136).

In 2007, Amato et al (137), found a significant correlation between cIMT and coronary atherosclerosis. A recent meta-analysis of 37000 individuals concluded that a 0.1mm increment of cIMT, after adjustment for age and sex, translated in a 10 – 15% increased risk of myocardial infarction and a 13 – 18% increased risk of having a stroke (138). Subsequently, cIMT is being increasingly used in research for risk stratification in individuals and as an end point in intervention studies. The wall

thickness can be measured at a single site, such as the far wall of the common carotid artery or at several sites including near and far walls of the left and right common carotid arteries, bifurcation, and internal carotid artery (139).

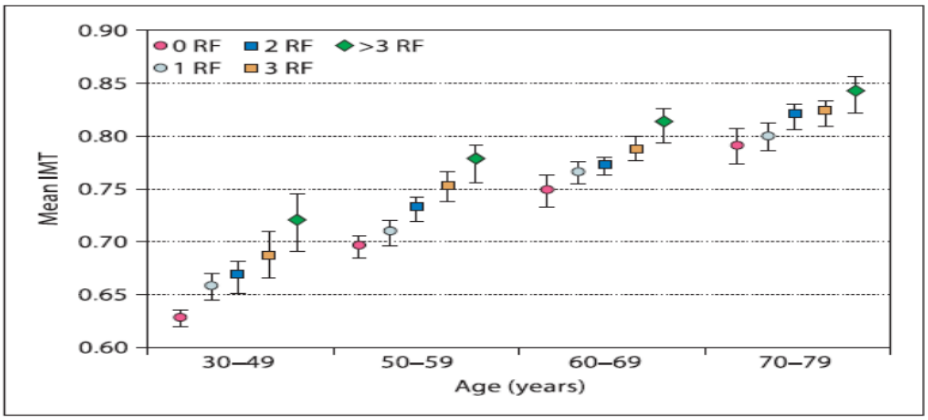


Figure 1.4 Correlation between cIMT and age-related quartiles of risk factors. Mean intima-media wall thickness (IMT) increases with person's age and the number of cardiovascular risk factors (RF) a person has including diabetes, smoking, hypertension, and elevated cholesterol levels. Obtained from Touboul et al, 2006 (131).

Difficulties that have been identified with cIMT testing include the high level of technical expertise needed for precise quantification (140), and the lack of standardisation of measurements and imaging protocols. It is not clear whether generalised cIMT or focal plaque formation is of more importance (141). The literature indicates that there are gender- and age-related differences with cIMT, and a definition of what are considered 'normal' limits that take into account these differences has not yet been established.

Despite the mixed literature regarding whether cIMT measurement provides benefit above traditional risk factors or if treatment guided by this test has an effect on clinical outcomes (142-146), cIMT has been accepted as an independent predictor of cerebral and coronary events by different societies including the American Heart Association (147), and European Society of Cardiology (148). It is thought that patients with documented subclinical atherosclerosis, as evidenced by cIMT, are at increased CV risk and may be considered candidates for more aggressive therapy.

1.2.2 cIMT in PCOS.

Data on the relationship between PCOS and cIMT are not conclusive. While some studies have shown PCOS to be associated with increased cIMT (126, 149), others did not (150, 151).

Orio et al. (126) examined 30 normal weight (BMI 22.4 ±2.1 kg/m^2) young (22.2 ±2.5 year) women with PCOS and compered them to age- and weight-matched controls. The authors showed PCOS was associated with increased cIMT 0.53 ±0.09 vs. 0.39 ±0.08 mm for normal controls. Both groups were normotensive and had no dyslipidaemia. The authors related the difference to increased IR and hyperandrogensim in the PCOS group. The cIMT results were adjusted for glucose and insulin levels, FAI, and waist-to-hip ratio (WHR).

On the other hand, Talbot et al. (150) studied the largest cohort of patients, 125 women with PCOS vs. 142 age-matched controls, and found no difference in cIMT between the two groups. On subgroup analysis, middle aged women with PCOS (≥45 years) had higher cIMT compared to weight and age matched controls 0.77 ±0.02 mm and 0.71 ±0.02 mm, respectively ($P<0.05$).

Treatment with metformin 850mg daily for six months, in the study by Orio et al. (152) resulted in a reduction in cIMT in the PCOS group, with cIMT measurements at six months similar to that seen in normal women, 0.53 ±0.09 vs. 0.40 ±0.07 mm, baseline vs. 6 months, respectively ($P<0.01$). The authors attributed the change to improvement in IR and to a lesser degree to improvement in hyperandrogenism, in particular an increase in SHBG and reduction in FAI. Interestingly, there was no control group and no drop-outs during this study.

While all PCOS women in Orio et al. study had oligomenorrhoea and clinical or biochemical evidence of hyperandrogenism (126), the PCOS group in Talbott et al.'s study were selected if they had evidence of chronic anovulation and either clinical/biochemical hyperandrogensim, or a ratio of LH to FSH of > 2 (150), suggesting that some of their study participants may not have fulfilled diagnostic criteria for PCOS (Table 1.1, page 4).

It is not yet clear if PCOS in younger women (age < 45 years), is associated with increased cIMT. It is also not known if weight loss would lead to improvement in cIMT in overweight women with PCOS.

1.3 Platelets.

1.3.1 Introduction.

Platelets are anucleated blood cells that originate from the cytoplasm of megakaryocytes in the bone marrow and circulate to 'survey' the integrity of the vascular system (153). Platelets exist in the circulation for 7 – 10 days and under normal conditions are discoid in shape with a surface that has extensive invaginations termed the open canulicular system (Figure 1.5a). These tiny folds may provide extra surface to enable platelets to spread out when activated (Figure 1.5b-d).

1.3.2 Platelet activation.

1.3.2 Platelet activation.

In healthy blood vessels, platelets circulate in a quiescent state close to the epithelial where they monitor cell wall integrity. Nitric oxide (NO) and prostacyclin (PGI_2) released by the endothelium moderate activation of platelets. Under normal conditions the influence of these inhibitors is more prominent than prothrombotic factors, also produced by the endothelium, including von Willebrand factor (vWF) and P-selectin (153, 154). In the event of endothelial damage to the blood vessel wall a chain of events is triggered leading to platelet-rich clot formation. Depending on the initiating event, this may represent normal haemostasis, for example wounds, or pathologic vascular thrombosis, as after the rupture of unstable atherosclerotic plaque in diseased arteries. The platelet mediated responses can be subdivided into four interrelated phases, adhesion, activation, secretion and aggregation (155).

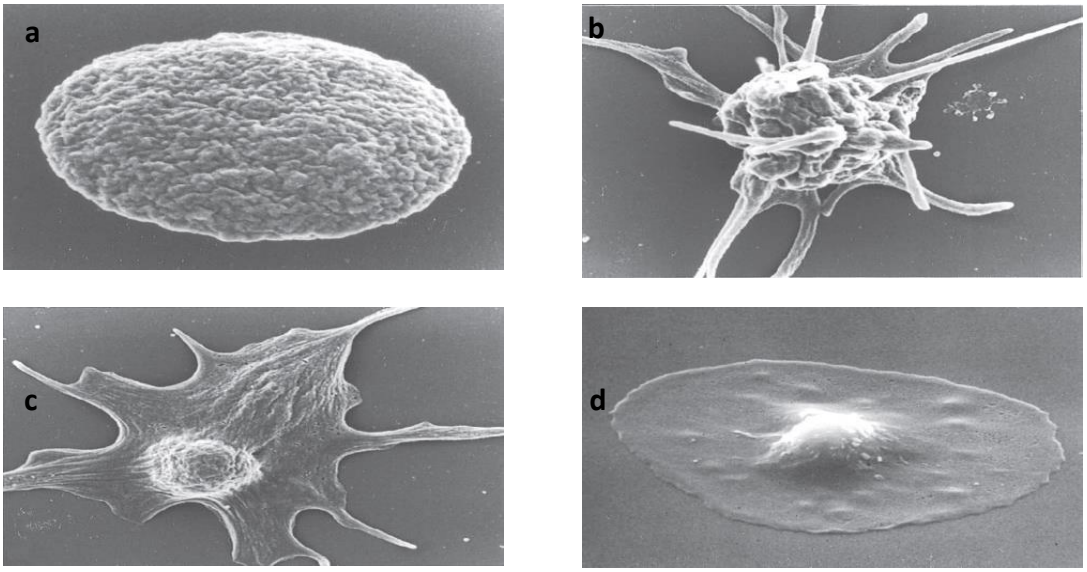


Figure 1.5 Platelet appearance at rest and activation. a) Discoid platelet photographed in the low-voltage, high-resolution scanning electron microscope. Magnification x30,000. b) Early dendritic platelet. Fine processes extend in all directions. Magnification ×13,000. c) Early spread platelet. The central body of the cell remains convoluted, but is gradually disappearing as the cytoplasm spreads and fills the spaces between pseudopods. Magnification ×11,000. d) Spread platelet. Magnification ×9000. Obtained from Alan D Michelson, 2007 (156).

An injury to the blood vessel wall leads to disruption of the endothelial cell lining and the exposure of prothrombotic extracellular matrix (ECM) proteins including vWF and collagen, which change the blood vessel wall from an anti-thrombogenic to a prothrombogenic phenotype. These ECM proteins capture and tether platelets from the flowing blood through receptor mediated processes (157, 158). Initial adhesion to collagen mainly occurs through glycoprotein (GP) VI; while adhesion to vWF is mediated by GPIbα on platelet membrane. Biochemical signalling events initiated by these proteins lead to the activation of integrin receptors $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ that bind to collagen and vWF, respectively and facilitate stable adhesion of platelets to the vessel wall through actin polymerization with cytoskeletal reorganization leading to shape change and pseudopod formation (Figure 1.4b-d). $\alpha_{IIb}\beta_3$ once activated is also able to bind plasma fibrinogen that can act as a molecular bridge between individual platelets and facilitate aggregation (159). These signalling events also lead to the release of platelet granules which act to

reinforce both haemostasis and vessel repair. Dense granules contain soluble platelet agonists, adenosine diphosphate (ADP) and adenosine triphosphate (ATP), which along with and thromboxane A_2 (T_XA_2), act in a paracrine fashion to further enhance platelet function and ensure rapid haemostasis activation (153). The release of a number of coagulation and growth factors from the α-granules promotes healing, while the expression of P-selectin, an α-granule protein, binds circulating neutrophils.

Activated platelets also provide a reactive surface for the activation of the coagulation cascade and the generation of thrombin. The serine protease thrombin cleaves platelet bound fibrinogen to form fibrin, which creates an insoluble meshwork around the platelet aggregates and prevents its dislocation by flowing blood. The steps described above are summarised in Figure 1.6.

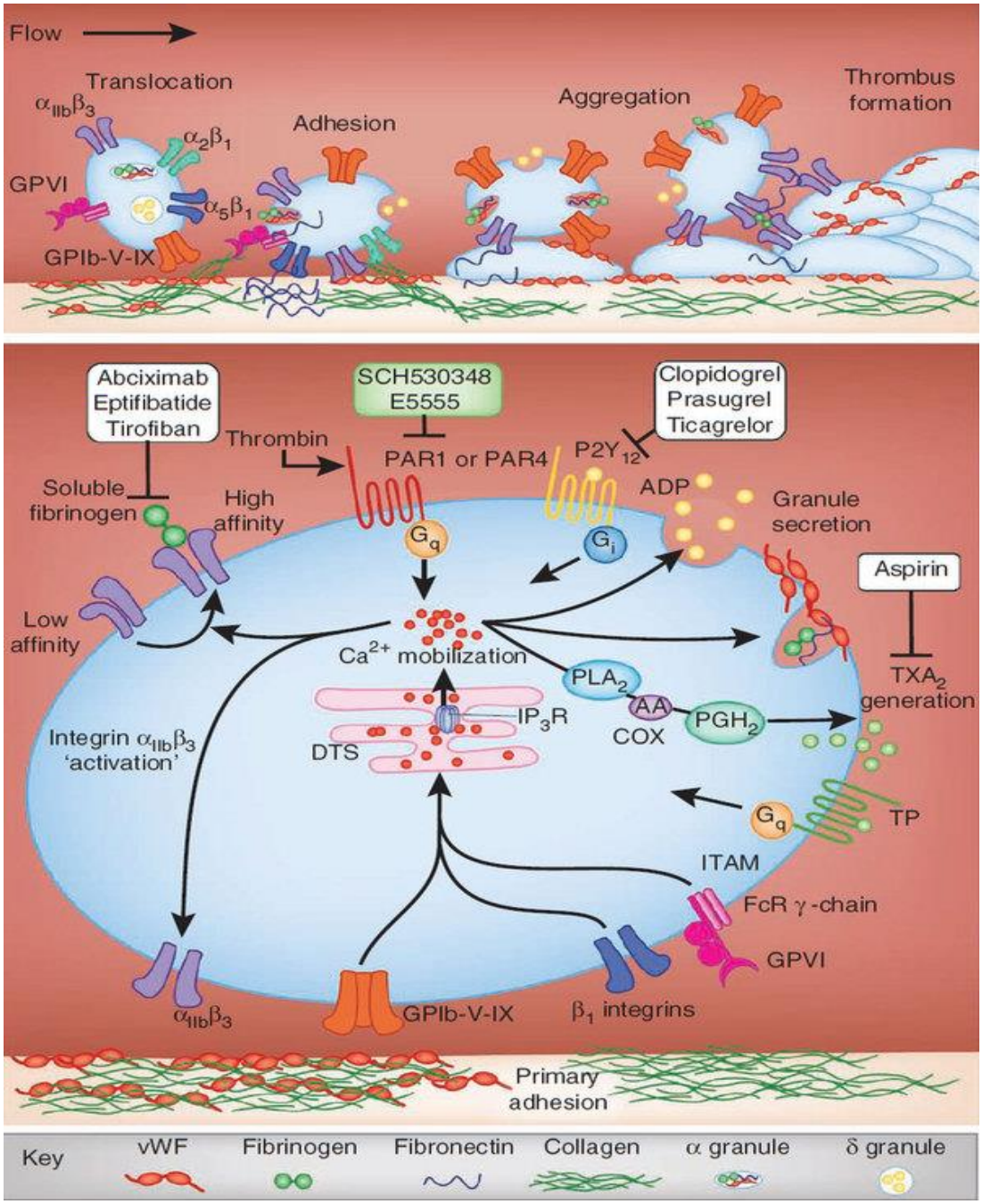


Figure 1.6 Adhesion and activation mechanisms supporting the haemostatic and prothrombotic function of platelets. Platelets are captured in the injured vessel wall from flowing blood via the specific interaction of the platelet GPIb-V-IX complex with collagen-bound vWF exposed on the subendothelium (top). This ligand-receptor interaction has a rapid on-off rate that supports platelet translocation at the vessel wall. Stable platelet adhesion occurs through the binding of platelet GPVI to fibrillar collagen as well as the ligation of multiple β1 integrins, including the collagen $\alpha_2\beta_1$ interaction and fibronectin engagement of $\alpha_5\beta_1$. Once

firmly adhered, platelets undergo a series of biochemical changes that induce integrin αIIbβ3 activation, leading to the high affinity interaction with adhesion proteins including vWF, fibrinogen and fibronectin. These adhesive interactions are indispensable in the ability of platelets to form stable aggregates with other activated platelets and promote thrombus growth. Activated platelets release or locally generate soluble agonists, including ADP, T_XA_2 and thrombin that can induce autocrine or paracrine platelet activation (bottom). Each agonist activates specific G protein‐coupled receptors on the platelet surface, including ADP-$P2Y_1$ or ADP-$P2Y_{12}$, T_XA_2-TP and thrombin‐human PAR1 or PAR4, stimulating intracellular signaling events that induce cytosolic calcium mobilization. This second messenger has a key role in promoting integrin $\alpha_{IIb}\beta_3$ activation, T_XA_2 generation, granule secretion and in the procoagulant function of platelets. While $P2Y_1$, not shown in the figure, is expressed in a wide range of cells, $P2Y_{12}$ has a more selective tissue distribution (160) making it an important target for platelet activation inhibitors. Currently approved antiplatelet agents for clinical use include; $P2Y_{12}$ receptor antagonists: clopidogrel, prasugrel and ticagrelor; TxA_2 production blockers: aspirin which irreversibly inhibit cyclooxygenase-1 activity; and GPIIb-IIIa inhibitors: abciximab, tirofiban, and eptifibatide which antagonise fibrinogen binding to $\alpha IIb\beta_3$ integrins. Antiplatelet agents currently in clinical trials include thrombin receptor antagonists: SCH530348 and E5555. Obtained from SP Jackson, 2011 (161).

1.3.3 Platelet agonists.

There is wide variety of physiological platelet agonists that differ in their ability to induce platelet activation. The most physiologically relevant agonists include thrombin, collagen, T_XA_2 and ADP is a weak agonist. These agonists seem to be important for different aspects of the thrombotic process.

1.3.3.1 Adenosine diphosphate.

ADP is released from the platelet dense granules upon activation and from red blood cells and damaged endothelial cells (162). The platelet response to ADP involves both a transient rise in free cytoplasmic calcium, mediated by the Gq-coupled $P2Y_1$ receptor, and inhibition of adenylyl cyclase, mediated by the Gi-

coupled $P2Y_{12}$ receptor (153, 160, 163). Coordinated signaling through each receptor is required for full platelet activation. ADP is a weak agonist that directly induces only shape change and reversible platelet aggregation, whereas the ensuing secretion and secondary aggregation are caused by the ADP-induced synthesis of T_XA_2 (153).

1.3.3.2 Thrombin.

Thrombin is a product of the coagulation cascade in which it is generated from its inactive precursor prothrombin. This reaction is greatly facilitated by the presence of activated platelets. Thrombin activates platelets in humans through G-protein protease-activated receptors PAR1 and PAR4 (164), and binding to the GPIbα subunit of the GPIb-IX-V complex (165). Thrombin interaction with its receptors results in several actions in platelets including an increase in calcium levels, activation of protein kinase C, T_XA_2 generation, and platelet aggregation (153).

1.3.3.3 Collagen.

Collagens are components of the extracellular matrix of the subendothelium. Type I and III collagens are among the most potent activators of platelets and support efficient platelet adhesion and aggregation under the rapid blood flow conditions that operate in stenosed arteries (161). Collagen interacts with platelet mainly through platelet membrane receptors $\alpha_2\beta_1$ (GPIa–IIa) and GPVI. Signaling involves tyrosine phosphorylation, SYK and phospholipase C activation (166).

1.3.4 Platelet inhibitors.

The main physiological platelet inhibitors include PGI_2 and NO; both are produced by the endothelium and act together to inhibit platelet activation (167).

1.3.4.1 Prostacyclin.

PGI_2 is a physiological platelet inhibitor produced by the endothelial cells predominantly through the conversion of arachidonic acid by cyclooxygenase-2 (COX-2) and prostacyclin synthase (168-170). PGI_2 binds to its G-protein coupled receptor (Gs) on platelet's surface and activates adenyl cyclase (AC) which mediates the production of cyclic adenosine monophosphate (cAMP) from ATP (167).

Elevated cAMP levels lead to the activation of Protein kinase-A (PKA) in platelets (167). PKA mediates the phosphorylation of several PKA substrates which alters several aspects of platelet function including the inhibition of cytoskeletal reorganization (shape change), intracellular calcium elevation and platelet secretion (171). A common PKA substrate is the vasodilator-stimulated phosphoprotein (VASP) and the main PKA phosphorylation site on VASP is ser^{157} (172). VASP plays a role in platelet shape change, adhesion, fibrinogen binding and aggregation (173, 174).

Phosphodiesterases (PDE) are enzymes that break down cAMP and control its function. Although eleven PDE isoenzymes have been discovered (175), only PDE3 is thought to play a significant role in platelet function (176). Inhibition of PDE3 results in increased PKA activity represented by enhanced phosphorylation of $VASP^{ser157}$ (176). Milrinone is a specific PDE3 inhibitor.

1.3.4.2 Nitric oxide.

NO is continuously produced by the vascular endothelium from L-arginine via NO synthase (NOS) (177). After release from the endothelium, NO diffuses across the platelet membrane and binds to the heme moiety of soluble guanylyl cyclase. This leads to guanylyl cyclase activation which then catalyses the production of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (178). cGMP activates cGMP-dependent protein kinase which results in inhibition of platelet activation by mechanisms including a decrease in intracellular calcium concentration, and in the expression of normal activation-dependent platelet surface glycoproteins, such as P-selectin and the active conformation of GPIIb‑IIIa (179, 180).

1.3.5 Platelets role in atherosclerosis.

The pathogenesis of atherosclerosis is multifactorial and platelets seem to play a role in acute thrombus formation at the site of vascular injury and contribute to atherogenesis. The role of platelets in the latter is not clear, but may contribute to the inflammatory processes and endothelial dysfunction (181).

The initial step in atherosclerosis is believed to be lipid deposition in the subendothelium, lipid oxidation and recruitment of leukocytes (182). This creates inflammation and altered endothelial cell function and potentially increased interaction between the endothelium and platelets (183). Platelets attached to an atherosclerotic lesion may influence plaque progression in different ways. For example, activated platelets bind to plasma molecules such as fibrinogen and express P-selectin, a protein released from α-granules and expressed on platelet surface only when activated. These interactions promote the adhesion of leukocytes to the vessel wall (153, 184). Platelet-leukocyte interaction through P-selectin leads to the release of chemokines and cytokines that promote atherosclerosis (184, 185). Circulating activated platelets and platelet-leukocyte/monocyte aggregates promoted the formation of atherosclerotic lesions in ApoE(-/-) mice (185). Apolipoprotein E (ApoE) is a ligand for receptors that clear chylomicrons and very low–density lipoprotein (VLDL) remnants (186). ApoE-deficient mice are popular in animal studies as they are at high risk of developing atherosclerotic lesions, even on chow diet, because of significantly increased circulating cholesterol levels (186). Higher levels of platelet P-selectin expression and fibrinogen binding have also been found to be higher in people with atherothrombotic diseases (187), obesity and diabetes (188), and associated with worse cardiovascular outcome after percutaneous coronary interventions (189). In addition, platelets also release growth factors; and may play a role in the metabolism of low-density lipoproteins in the atherosclerotic lesion (190).

1.3.6 Platelet function in PCOS.

While platelet function in PCOS is ill defined, emerging data suggest the presence of platelet hyperactivity. The first paper to suggest platelet dysfunction in PCOS was published by Dereli et al. 2003 (191). Lean women with PCOS (n=50) were compared to women with NC-CAH (n=50), and normal controls (n=30). Only lean women with PCOS were chosen to avoid the effect of obesity on IR. All women with PCOS had clinical and/or biochemical hirsutism in addition to oligomenorrhoea (<6 periods/year) or anovulation. Women with PCOS had higher IR, measured using homeostasis model assessment for insulin resistance (HOMA-IR). Free testosterone

levels were not different between PCOS and NC-CAH groups but were significantly higher than controls (191). Platelet aggregation, measured in platelet rich plasma (PRP), in response to ADP, collagen or epinephrine was higher in PCOS women compared to the other two groups for all three agonists. However platelet aggregation in women with NC-CAH was similar to controls (191). The authors suggested that platelet dysfunction in PCOS was secondary to increased IR rather than hyperandrogensim, as the NC-CAH group had similar platelet aggregation compared to controls (191).

In a recent paper by Rajendran et al. (125) platelet function in a group of lean healthy women (n=12) was compared to lean PCOS (n=12) and obese PCOS (n=12). Platelet aggregation in response to ADP and their responsiveness to NO were measured in whole blood. Women with PCOS were diagnosed according to the Rotterdam Criteria (7), while women in the normal control group had normal menses and no history of hirsutism or hyperandrogensim. The three groups were matched for age and smokers were excluded. There was no difference in IR, measured using HOMA-IR and Quiki, and BMI between lean PCOS and controls. Lean and obese PCOS had higher testosterone, and malondialdehyde (MDA), a marker of oxidative stress, compared to controls (125). ADP induced platelet aggregation was elevated, and platelet responsiveness to the inhibitory effects of NO was lower in lean and obese PCOS compared to controls. There was no difference in ADP-induced platelet aggregation and responsiveness to NO between lean and obese women with PCOS. Rajendran et al. suggested that hyperandrogensim is likely to be responsible for platelet dysfunction in PCOS, especially as lean women with PCOS had no significant difference in BMI or IR compared to controls (125).

It is interesting that although both papers discussed above suggest impaired platelet function in PCOS, there was no consistency with regards to their explanation of the underlying mechanism, i.e. IR vs. hyperandrogenism. In addition, the study by Rajendran et al. (125) did not account for obesity. As we have discussed previously, obesity may play an important role in the aetiology of PCOS. Platelet function has been found to be impaired in obesity with reduced platelet

sensitivity to NO and PGI_2 in aggregation (192) and increased urinary T_XA_2 metabolite excretion (193). Although current knowledge suggests platelet dysfunction in PCOS, it remains unclear if this is independent of obesity or not, and whether weight loss alters platelet function in obese women with PCOS. While lifestyle modifications are recommended for weight loss, their efficacy is limited by low compliance rate (91). Recently, an incretin (explained below) analogue, liraglutide, was found to cause weight loss in healthy overweight men and women and people with T2DM (114, 194, 195). Liraglutide's effect on platelet function is unknown.

1.4 Incretins.

1.4.1 Introduction.

The gut produces and harbours a number of different biologically active peptides. The possibility that intestinal factors are secreted in response to nutrients which can lower blood glucose levels was described in the early 1900s (196, 197). These factors were named 'incretins' in the 1930s (197).

The incretin effect was first described by Elrick et al. (198) in 1964 following the observation that insulin responses to oral glucose exceed those measured after intravenous administration of equivalent amounts of glucose (Figure 1.7). The incretin effect in healthy individuals is responsible for 50–70% of the insulin response to a meal (199).

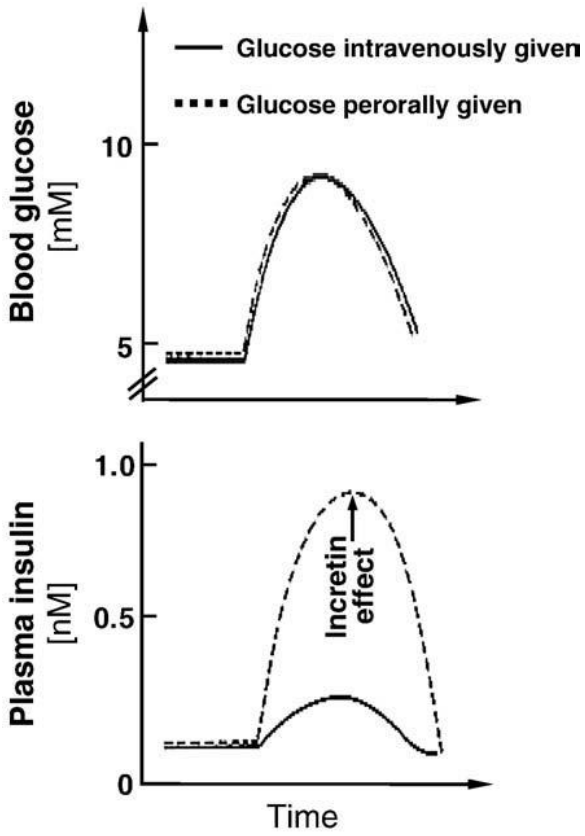


Figure 1.7 The incretin effect. Note the difference in plasma insulin following oral and intravenous glucose administration. Plasma insulin levels were significantly higher when glucose was given orally compared to intravenously. Obtained from EJ Verspohl, 2009 (200).

The most important incretins are glucose-dependent insulinotropic polypeptide (GIP), and GLP-1 (201). Gene deletion of the GIP receptor or the GLP-1 receptor in mice results in impaired insulin secretion after oral glucose in conjunction with glucose intolerance (202, 203).

1.4.2 Glucose-dependent Insulinotropic Peptide.

GIP was the first incretin to be described. It is a single 42 amino acid peptide derived from a 153 amino acid precursor encoded by a gene located on chromosome 17 (204, 205). It is secreted in a single bioactive form from the K-cells in the duodenum and jejunum in response to the ingestion of carbohydrates and/or lipids (197, 204, 205). GIP results in glucose-dependent insulin secretion in humans (197, 205, 206). In addition, it plays a role in fat metabolism in the adipocytes and has a proliferative effect on the β-cells (205, 207, 208).

1.4.3 Glucagon like Peptide-1.

This was the second incretin to be discovered. GLP-1 is a 30 amino acid peptide that is produced in the intestinal L cells, located mainly distally in the small intestine (209). GLP-1 is encoded by the proglucagon gene localized to band q36-q37 on chromosome 2 (210). It is processed from its precursor or prohormone, proglucagon, through the action of proconvertase 1/3. Although GLP-1 and glucagon are encoded in the same gene, GLP-1 and glucagon are not produced by the same cells. GLP-1 is produced in the gut, whereas glucagon is produced in the pancreatic islets. This is due to tissue-specific expression of the proconvertases, because proconvertase 1/3 is not expressed under normal conditions in the islet α cells. Instead, α cells express proconvertase 2, which processes proglucagon to glucagon (Figure 1.8) (197, 205, 211).

Despite the distal location of the L-cells, GLP-1 is secreted within minutes following oral intake which suggests that neural and endocrine factors rather than direct stimulation are involved (197, 205). These factors are not well understood in humans but animal models suggest a role for taste receptors and vagal stimulation (197, 205, 212, 213).

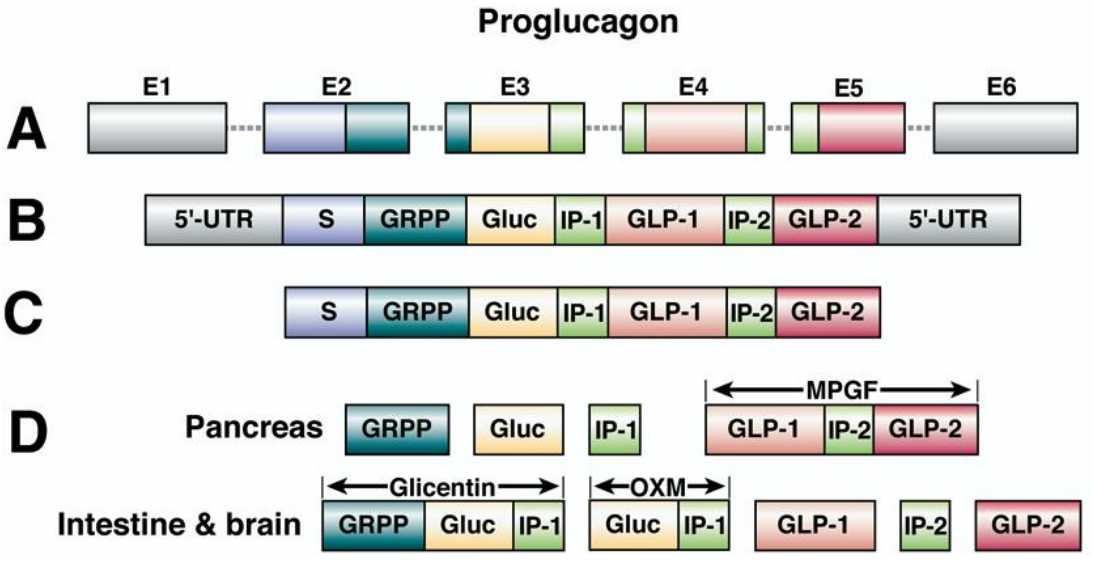


Figure 1.8 Production of GLP-1 from proglucagon gene. Structures of (A) the proglucagon gene, (B) mRNA, and (C) proglucagon protein. (D) Tissue-specific posttranslational processing of proglucagon in the pancreas, through the actions of proconvertase 2, leads to the generation of Glicentin-related polypeptide (GRPP),

glucagon (GLUC), intervening peptide-1 (IP-1), and major proglucagon fragment (MPGF), whereas glicentin, oxyntomodulin (OXM), intervening peptide-2 (IP-2), and GLP-1 (glucagon like peptide-1) and GLP-2 (glucagon like peptide-2) are liberated after proglucagon processing in the intestine and brain through the action of proconvertase 1/3. Obtained from LL Baggio and DJ Drucker, 2007 (197).

Following its release into the circulation, GLP-1 is rapidly degraded to its inactive form by removal of the two N-terminally located amino acids (214). This process is executed by the enzyme dipeptidyl peptidase IV (DPP-IV) (Figure 1.9), which is a postproline cleaving serine protease. DPP-IV is both a cell surface protein and a circulating protein. DPP-IV is widely expressed in human tissues including the brain, lungs, kidneys, adrenals, pancreas, intestine and lymphocytes (205). Interestingly, it is found in the endothelial cells of the blood vessels that drain the intestinal mucosa where the L-cells are situated (205, 215). This suggests that the majority of GLP-1 is inactivated almost immediately following secretion. This rapid inactivation of GLP-1 and GIP contributes to a half-life of less than 2 min and 5–7 min respectively (197, 205, 216, 217). This raises the question of whether significant amounts of intact, bioactive, peptide really reach the arterial circulation and target tissues; subsequently, some uncertainty exists over how GLP-1 mediates its actions (200).

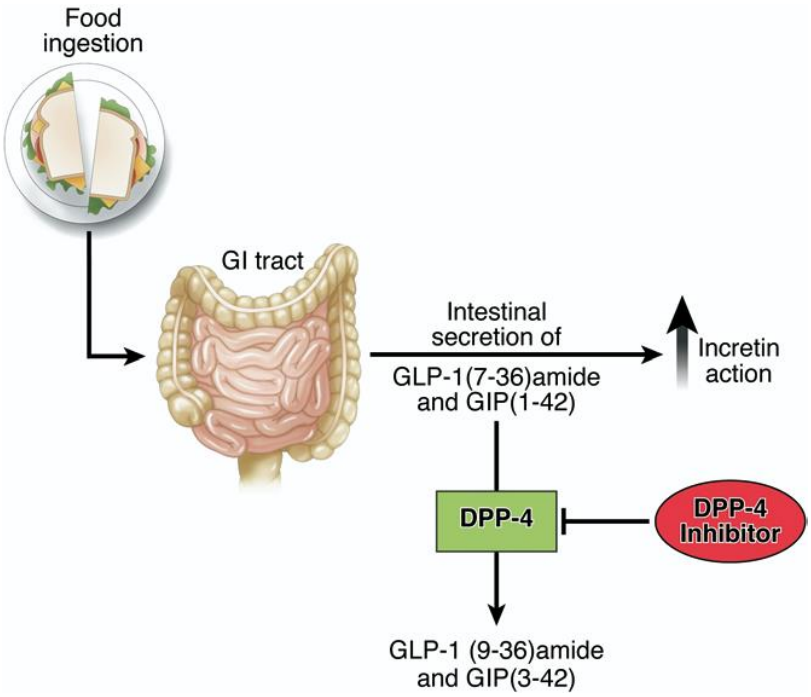


Figure 1.9 Inactivation of GLP-1 and GIP by DPP-IV. Bioactive GLP-1 (7-36) amide and GIP (1-42) are released from the small intestine after meal ingestion and enhance glucosestimulated insulin secretion (incretin action). DPP-4 rapidly converts GLP-1 and GIP to their inactive metabolites GLP-1 (9-36) and GIP (3-42) in vivo. Inhibition of DPP-4 activity prevents GLP-1 and GIP degradation, thereby enhancing incretin action. GLP-1, glucagon like peptide-1; GIP, glucose-dependent insulinotropic peptide; DPP-4, dipeptidyl peptidase IV (DPP-IV). Obtained from LL Baggio and DJ Drucker, 2007 (197).

Unlike GIP, GLP-1 suppresses glucagon secretion from α-cells in the pancreas, gives a feeling of satiety, and reduces food intake, gastric emptying and body weight (Figure 1.10) (205). Although GIP levels in subjects with T2DM are either normal or increased, the acute insulinotropic response to native GIP is diminished substantially (218). In contrast, while meal-stimulated plasma levels of GLP-1 are usually reduced in subjects with T2DM (219), the glucoregulatory actions of GLP-1 are preserved (218). Although the exact mechanisms underlying the reduced response to GIP remain unclear, preclinical studies suggest that hyperglycaemia may be associated with down-regulation of GIP receptor expression in rodent islets

191. Hence, GLP-1 has attracted more interest as a target for therapeutic development for the treatment of T2DM (219-221).

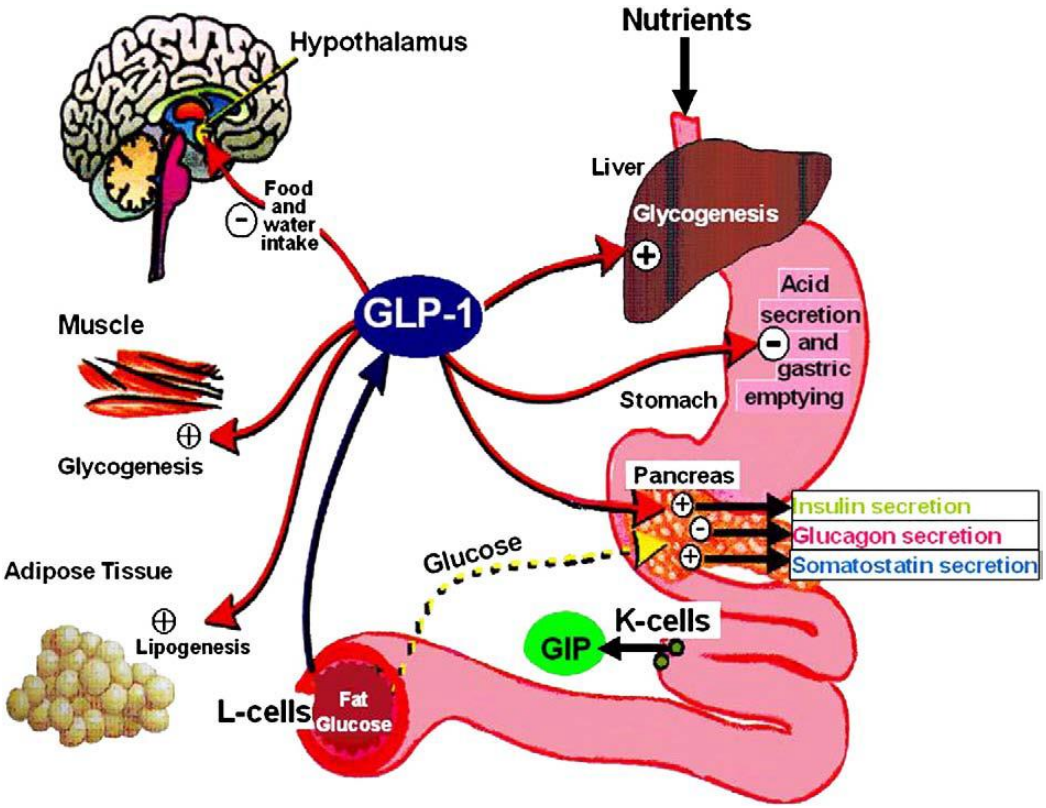


Figure 1.10 Physiology of GLP-1 secretion and action on various tissues. Glucagon like peptide-1 (GLP-1) is produced from the L-cells of the small intestine in response to food ingestion. Glucose-dependent insulinotropic peptide (GIP) is produced from the K-cells of the duodenum and jejunum in response to food ingestion. The pleiotropic effects of GLP-1 include the brain (satiety), peripheral organs (increases glycogenesis in the liver and muscles, increases lipogenesis in the adipose tissue and reduces gastric emptying), and endocrine pancreas (increase of insulin and somatostatin release and inhibition of glucagon release). Obtained from E.J. Verspohl, 2009 (200).

1.4.3.1 Mechanism of action of GLP-1.

GLP-1 is widely believed to exert its actions through a distinct heptahelical G protein-coupled receptor (GLP-1 receptor), which is functionally associated with adenylate cyclase through the stimulatory Gs (222, 223). GLP-1R is found in the pancreas, stomach, skeletal muscle, heart, lung and brain (211, 224). GLP-1 may increase intracellular cAMP and activate PKA, thereby increasing insulin secretion in beta cells. GLP-1 is also thought to activate the PI3K/Akt pathway through epidermal growth factor receptor via GLP-1 receptor activation in beta cells (225).

1.4.3.1.1 GLP-1 regulation of β-cell function.

The resting membrane potential of the β-cell is principally determined by the ATP-sensitive K^+ channels (K_{ATP} channels) (226-228). In the unstimulated β-cell, the K_{ATP} channels are spontaneously active and the membrane potential of the cell is therefore "clamped" near the K^+ equilibrium potential, i.e. between –70 mV and –80 mV (227-229). When the blood glucose concentration rises, it equilibrates rapidly across the β-cell membrane (effect of the GLUT2 glucose transporter system) and glucose metabolism is accelerated. This in turn leads to increased generation of ATP at the expense of ADP and the cytoplasmic ATP:ADP ratio increases, leading to the closure of the K_{ATP} channels (230-232). Once the K_{ATP} channels are sufficiently inhibited (>90%), the remaining K^+ permeability is no longer sufficient to balance the depolarising influence. This membrane depolarisation culminates in the activation of voltage-dependent L-type Ca^{2+} channels, the initiation of Ca^{2+} dependent action potentials, Ca^{2+} influx and exocytosis of the insulin-containing granules (Figure 1.11).

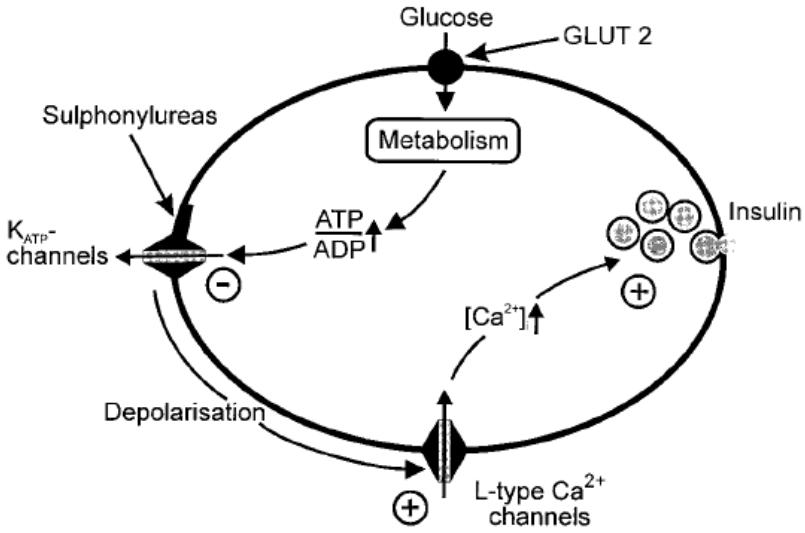


Figure 1.11 Model for the glucose-stimulated insulin secretion of the pancreatic β-cell. As blood glucose levels rise, glucose enters the pancreatic β-cell through the glucose transporter GLUT2. Glucose metabolism leads to an increase in adenosine triphosphate (ATP) levels, at the expense of adenosine diphosphate, and closure of the ATP-sensitive K^+ channels (K_{ATP} channels), with subsequent depolarisation of the resting membrane of the β-cell. Membrane depolarisation leads to activation of the L-type calcium (Ca^{2+}) channels with subsequent influx of calcium into the β-cell, which potentiates insulin exocytosis and secretion from its stores. Obtained from Gromada et al. 1998 (233).

Patch-clamp studies on isolated rat β-cells have suggested that GLP-1 reversibly inhibits K_{ATP} channels in a glucose-dependent fashion (234). Similar observations have been made subsequently in both mouse and human β-cells (235, 236). The synergistic action of glucose and GLP-1 has dual effects on β-cell electrical (and secretory) activity. First, electrical activity may be initiated when GLP-1 is applied at glucose concentrations which are themselves sub-threshold. Secondly, the electrical activity is increased in islets already exposed to a stimulatory glucose concentration (235, 236).

The action of GLP-1 on the K_{ATP} channel activity is reversed by the specific PKA inhibitor adenosine-3',5'-cyclic monophosphorothionate, Rp diastereomer (Rp-cAMPS), suggesting that the effects are secondary to the generation of cAMP and

activation of PKA (234, 235). Activated PKA acts synergistically with glucose to close K_{ATP} channels and thus facilitates membrane depolarisation and the induction of electrical activity. The effects of activated PKA are possibly mediated through the phosphorylation of K_{ATP} channel components (KIR6.2 and SUR1) which facilitates the binding of ATP to the inhibitory site of the K_{ATP} channel and its subsequent closure (237-239). In addition, activated PKA may also stimulate glucose metabolism leading to increased ATP levels (233).

GLP-1 causes a rise in Ca^{2+} in both isolated β-cells and in various insulinoma cell lines. This increase is secondary to activated PKA and the phosphorylation of voltage-dependent Ca^{2+} channels with subsequent stimulation of Ca^{2+} influx and is not observed in the presence of L-type Ca^{2+} channel antagonists or following removal of Ca^{2+} from the extracellular medium (240-244).

Calcium influx across the plasma membrane would also lead to the mobilisation of Ca^{2+} from intracellular ryanodine- and inositol trisphosphate (IP3)-sensitive stores in what is known as the Ca^{2+}-induced Ca^{2+} release. In addition, activated PKA promotes the mobilization of Ca^{2+} from intracellular stores and the generation of IP3 (245). The ability of GLP-1 to mobilise intracellular Ca^{2+} in voltage clamped mouse β-cells is glucose-dependent, with no effect of the peptide being observed at a sub-stimulatory concentration of glucose (5 mM) (245). The strong insulinotropic actions of GLP1 in the β-cell are summarised in (Figure 1.12).

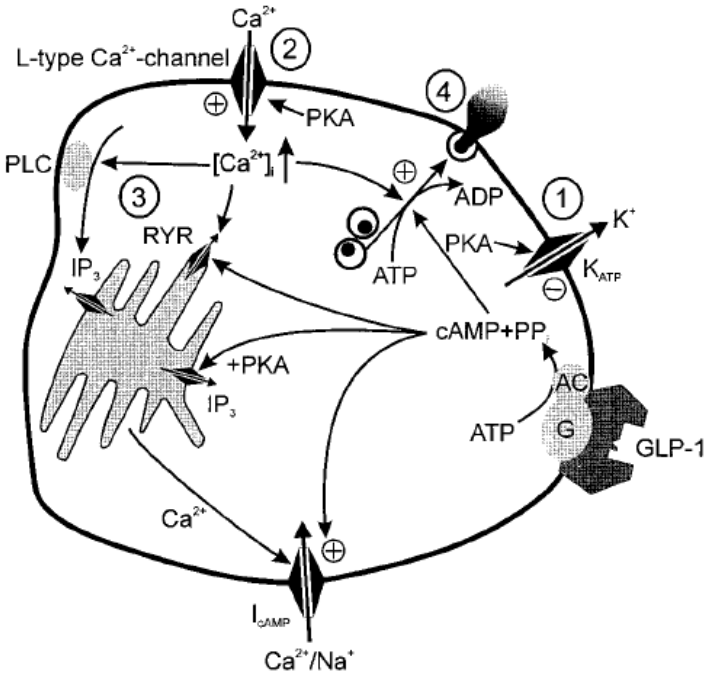


Figure 1.12 Summary of the cellular actions of GLP-1 that lead to stimulation of insulin secretion. Binding of glucagon like peptide-1 (GLP-1) to its G protein-coupled receptor activates adenylyl cyclase (AC) which results in an increase in cyclic adenosine monophosphate (cAMP) levels and subsequent protein kinase A (PKA) activation. Activated PKA facilitates the closure of K_{ATP} channel [1] and the increase in intracellular calcium (Ca^{2+}) levels by stimulating Ca^{2+} influx from outside the cell by L-type Ca^{2+}-channel [2], and the release of Ca^{2+} from intracellular stores. Calcium influx may also stimulate the release of Ca^{2+} from intracellular stores [3]. These events will result in insulin secretion [4]. ATP, adenosine triphosphate; ADP, adenosine diphosphate; IP3, inositol trisphosphate; PLC phospholipase C, RYR ryanodine receptors. Obtained from Gromada et al. 1998 (233).

1.4.3.1.2 GLP-1 induces β-cell growth.

Evidence suggest that GLP-1 increases in vitro β-cell proliferation non-additively with glucose via a phosphatidylinositol (PI) 3-kinase/protein kinase C signalling pathway in pancreatic (INS-1) cells (246, 247), and increases islet mass in mouse pancreas in vivo (248). However, the effects of GLP-1R activation on β-cell mass expansion are not very well understood and likely to involve multiple signal transduction pathways including transactivation of the epidermal growth factor

receptor (EGRP) (225), insulin-receptor substrate-2 (IRS-2) signaling (249), and activation of cAMP/PKA, PI-3K, and mitogen-activated protein kinase (MAPK) signaling (197). A shared component in all GLP-1R–dependent molecular pathways involved in β-cell mass expansion and cytoprotection seems to be the activation of the transcription factor pancreas duodenum homeobox-1 (Pdx-1) (197, 250).

1.4.4 Liraglutide.

1.4.4.1 Structure and pharmacology.

Native GLP-1 is a 30-amino acid peptide produced by cleavage of the transcription product of the preproglucagon gene (251). Liraglutide was obtained by substitution of Lys 34 to Arg, and by addition of a C16 fatty acid at position 26 using a Ɣ-glutamic acid spacer (Figure 1.13).

Structure-activity data supporting the specific modifications in the liraglutide molecule were reported by Knudsen et al. (252) and Madsen et al. (253) Knudsen et al. varied the position of attachment of the Ɣ-glutamic acid spacer and C16 fatty acid, achieving half-lives of up to 20 hours (252). Madsen et al. prepared a series of analogues with varying lengths and structures of fatty acid moiety, which protracts the circulating lifetime of acylated peptides by binding to albumin (253). Their data indicated a close relationship between fatty acid length and half-life, ranging from 0.8 hour for a C10 fatty acid to 21 hours for a C18 fatty acid. Potency for the GLP-1 receptor was unaffected by chain length for fatty acids up to C16, but was reduced for the analogue with C18 fatty acid.

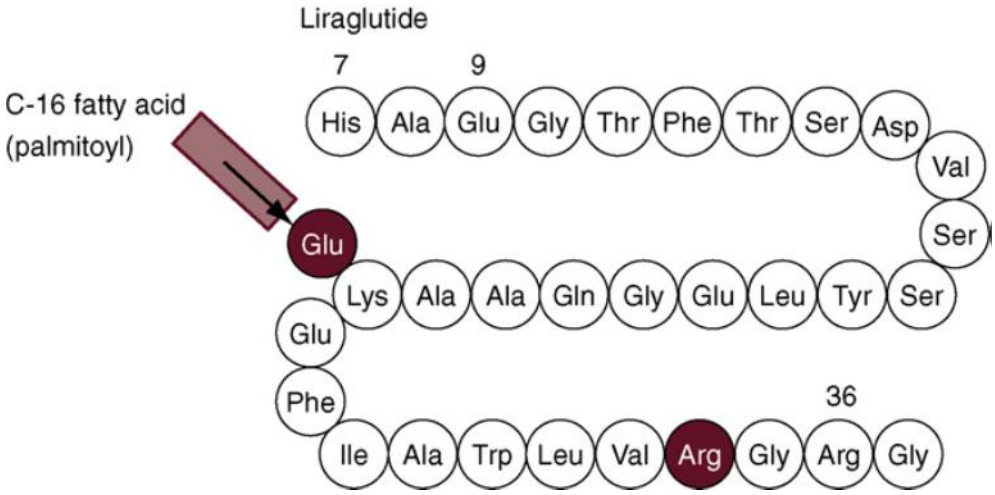


Figure 1.13 Structure of liraglutide. Liraglutide is a glucagon like peptide-1 (GLP-1) analogue. It differs from GLP-1 by the substitution of Lys 34 to Arg, and by the addition of a C16 fatty acid at position 26 using a ɣ-glutamic acid spacer. Obtained from Russell-Jones, 2009 (254).

Liraglutide is administered as an isotonic solution by subcutaneous injection. Pharmacokinetics of liraglutide are suitable for once-daily administration, with a Tmax of 9–13 hours and a T1/2 of 13 hours (255). This increase in the half-life of liraglutide is most likely to be mediated via a lower susceptibility to metabolism by DPP-IV (253) and a high degree of albumin binding of liraglutide (256, 257), and slow absorption of liraglutide after subcutaneous administration, as evidenced by the further increase in half-life observed with subcutaneous vs. intravenous administration. Thus, liraglutide treatment probably induces much higher plasma levels compared with the native GLP-1 concentration in plasma (258-260). Average steady state concentration over 24 hours is approximately 128 ng/mL for a dose of 1.8mg/day (255). The 24 hours duration of action was confirmed by Degn et al. (261) who found a sustained glucose lowering effect 24 hours after the last dose of liraglutide at steady-state achieved after three doses.

1.4.4.2 Mechanism of action.

Similar to native GLP-1, liraglutide induces insulin secretion from β-cells via a specific interaction with GLP-1R, leading to an increase in cAMP (255). Liraglutide

stimulates insulin secretion in a glucose-dependent manner. Simultaneously, liraglutide lowers inappropriately high glucagon secretion.

Liraglutide may also work through a GLP-1R-independent pathway. Hattori et al. suggested that the effects of liraglutide on endothelial cells are at least partly mediated through activation of adenosine monophosphate-activated protein kinase (AMPK) and in a signaling pathway independent of the GLP-1R. Liraglutide, dose-dependently increased nitric oxide production in human umbilical endothelial cells (HUVECs) (262). eNOS phosphorylation was seen to follow the same time course as AMPK activation. eNOS phosphorylation by liraglutide was attenuated by the AMPK inhibitor compound C, but not by the PI3K/Akt inhibitor LY2294002 or the PKA inhibitor KT5720 (262).

1.4.4.3 Clinical efficacy.

The effects of liraglutide in people with T2DM, as a monotherapy and in combination with other anti-diabetic medications, was investigated in a series of 6 randomized controlled trials, LEAD trials, that involved more than 4400 patients with T2DM, of which 2700 approximately received liraglutide (160). Liraglutide improved glycosylated haemoglobin A1c (HbA1c), fasting plasma glucose and postprandial glucose compared with placebo. Favourable effects were also noted on weight and systolic blood pressure (115, 154, 156-159).

The clinical effect of liraglutide as a weight loss medication was investigated in a 20-week randomised, double-blind, placebo controlled study (114). 564 individuals (18–65 years of age, BMI 30–40 kg/m²) were randomly assigned to one of four liraglutide doses (1.2 mg, 1.8 mg, 2.4 mg, or 3.0 mg, n= 90–95) or to placebo (n= 98) administered once a day subcutaneously, or orlistat (120 mg, n=95) three times a day orally. All individuals had a 500 kcal per day energy-deficit diet and increased their physical activity throughout the trial. Mean weight loss with liraglutide 1·2mg, 1.8mg, 2.4mg and 3.0 mg was 4.8 kg, 5·5 kg, 6.3 kg, and 7.2 kg, respectively; compared with 2.8 kg with placebo and 4·1 kg with orlistat (114). Despite its effect on weight, liraglutide is currently only approved for the treatment of people with T2DM (162, 263).

1.4.4.4 Side effects.

Overall, liraglutide is generally well tolerated, with most adverse events across the LEAD studies reported to be gastrointestinal-related. Nausea was the most common adverse event reported affecting up to 40% of patients at the start of treatment and resolved in the majority within four weeks (115, 154, 156-159). There were no major hypoglycaemic episodes on liraglutide monotherapy. The incidence of withdrawals due to adverse reactions was 7.8% with the most frequent adverse reaction leading to withdrawal being nausea (2.8% of patients) and vomiting (1.5%) (264).

A few cases (<0.2%) of acute pancreatitis have been reported during long-term clinical trials with liraglutide (264). Non-lethal thyroid C-cell tumours were seen in 2-year carcinogenicity studies in rats and mice. These tumours were not seen in monkeys treated for 20 months. These findings in rodents are caused by a non-genotoxic, specific GLP-1 receptor-mediated mechanism to which rodents are particularly sensitive. The relevance for humans is likely to be low but cannot be completely excluded (264).

1.4.4.5 Effects of liraglutide on cardiovascular risk.

Pre-clinical trials suggest that liraglutide may have a cardioprotective effect (265). GLP-1R was found to be expressed throughout the mouse cardiovascular system (266). Noyan-Ashraf et al. (265) treated normal and diabetic mice with liraglutide for seven days before inducing myocardial infarction. Survival was significantly higher in the liraglutide treated mice, independent of weight loss. Liraglutide reduced cardiac rupture and infarct size and improved cardiac output (265). It is not known if platelets express GLP-1R; or if treatment with liraglutide affects CV risk in humans.

1.5 Summary and project aims.

PCOS is the most common endocrine disorder of women of reproductive age. Obesity is strongly linked to PCOS and carries increased CV risk. Studies investigating atherothrombotic risk in women with PCOS, in particular platelet function and cIMT, have been confounded by not adequately accounting for obesity. Liraglutide is a GLP-1 analogue that causes weight loss and has been found to have a favourable effect on atherothrombotic risk in preclinical and animal studies. Subsequently, liraglutide may represent an attractive option for the treatment of obesity in women with PCOS, although clinical data are lacking. In particular, the effects of treatment with liraglutide on atherothrombotic risk, liver fibrosis markers and quality of life in young obese women with PCOS are not known. In addition, it is not known whether platelets express the GLP-1R or if liraglutide has a direct effect on platelet function.

The work conducted during this PhD aimed to answer the following questions:

1. Is atherothrombotic risk, in particular platelet function and cIMT, increased in young obese women with PCOS independent of obesity?
2. Is GLP-1R expressed in human platelets and does liraglutide have a direct effect on platelet function?
3. Do obese women with PCOS and controls respond equally to treatment with liraglutide?
4. Does treatment with liraglutide alter atherothrombotic risk, liver fibrosis markers and quality of life in young obese women with PCOS and controls?

2. Chapter 2: The effects of polycystic ovary syndrome on platelet function and atherothrombotic risk in obesity.

2.1 Abstract.

Previous studies investigating cardiovascular (CV) risk in obese women with polycystic ovary syndrome (PCOS) have been potentially confounded by not adequately accounting for body weight. The objective of this study was to assess if PCOS increases CV risk independently of obesity in young women by examining carotid intima-media wall thickness (cIMT) and platelet function. We conducted a case-control study comparing women with PCOS (n=21) to age (32.8 ±7.2 vs. 33.5 ±6.7years), and weight (100.9 ±16.7 vs. 99.3 ±14.7kg) matched controls (n=19). Platelet function was examined by flow cytometry, clot structure and fibrinolysis by turbidimetric assays and endothelial function by ELISA and post-ischaemic reactive hyperemia. The PCOS group had higher testosterone 1.2 ±0.3 vs. 0.9 ±0.3nmol/L (P=0.01), HOMA-IR 5.1 ±2.6 vs. 3.5 ±1.3 (P=0.03), impaired glucose regulation 33.3% vs. 5.3% (P=0.02), compared to controls. Mean cIMT 0.5 ±0.05 vs. 0.48 ±0.06 mm (P=0.36), and basal platelet surface expression (percentage of positive cells) of P-selectin 0.52 ±0.3 vs. 0.43 ±0.23 (P=0.40) and fibrinogen binding 0.97 ±0.4 vs. 0.83 ±0.3 (P=0.48) did not significantly differ between the PCOS and control groups, respectively. Furthermore, platelets sensitivity to adenosine-5'-diphosphate (ADP) or inhibition with prostacyclin, clot structure and fibrinolytic efficiency *ex vivo*, endothelial reactive hyperemic index (RHI), inflammation (hsCRP) and adhesion markers (sE-selectin, sP-selectin, sVCAM-1 and sICAM-1) were not significantly different between the two groups. In conclusion, PCOS appeared not to independently increase atherothrombotic risk when matched for obesity. It is likely that any excess CV risk in young obese women with PCOS can either be attributed to obesity or is not yet apparent at young age.

2.2 Introduction.

Polycystic ovary syndrome (PCOS) is often associated with obesity (75) and cardiovascular (CV) risk markers (5). Obesity and atherosclerosis are associated with a number of atherothrombotic indices including impaired vascular response to post-ischaemic reactive hyperemia (267), and elevated levels of serum endothelial adhesion markers sE-selectin, sP-selectin, intercellular adhesion molecule-1 (sICAM-1), and vascular cell adhesion molecule-1 (sVCAM-1) (268). PCOS, independent of obesity, is associated with insulin resistance (IR), increased risk of type 2 diabetes (T2DM), and presumed increased CV risk (5). However, long term CV outcome data in women with PCOS is lacking and it remains unclear whether PCOS independently increases atherothrombotic risk or CV morbidity and mortality (5).

Carotid intima-media wall thickness (cIMT), measured by brightness mode ultrasonography, detects early signs of atherosclerosis (131). Higher mean cIMT is associated with increased risk of myocardial infarction, stroke, vascular death, and total mortality (269). Data on the relationship between PCOS and cIMT are not conclusive (126, 150). In particular, it is not clear if PCOS in younger women (age <45yr) is associated with increased cIMT or whether PCOS influences the atherogenic process in obesity (126, 150).

Atherothrombotic disease is also a hypercoagulable state driven by abnormalities in platelet and haemostatic function (153). Dense fibrin clots, with thin fibers and small pores, are resistant to fibrinolysis and have been found in people with CV disease (270). Platelet function has been found to be impaired in obese women with PCOS when compared to lean controls (125). Obesity and atherosclerosis are associated with reduced platelet sensitivity to nitric oxide (NO) and prostacyclin (PGI_2) in aggregation (described in Chapter 3, paragraph 3.4.3) (192), and increased platelet surface expression of P-selectin (CD62P) and fibrinogen binding in flow cytometry (187). IR, inflammation and oxidative stress are thought to play a role in platelet dysfunction in obesity (193, 271). It remains unclear whether PCOS augments the effect that obesity may have on platelet function and thrombotic risk, and if this relates to increased IR, and/or hyperandrogenism, commonly found in PCOS.

The primary aim of this study was to assess whether cIMT is increased and platelet function is altered in women with PCOS independent of obesity. The study's secondary aims were to examine other markers of atherothrombosis including clotting function, and inflammation. We hypothesised that PCOS independently increases atherothrombotic risk in obesity.

2.3 Participants and methods.

2.3.1 Study design.

A case-control study of young women with PCOS and age- and weight-matched controls.

2.3.2 Ethics approval.

This was obtained before initiating the study. An ethics application form was submitted through the Integrated Research Application System (IRAS), which is a single online system for applying for permissions and approvals for health and social care/community research in the United Kingdom (UK) (272). After successful submission of the application, the study was discussed at the National Health Service (NHS) Leeds (East) Research Ethics Committee (REC) meeting. I attended this meeting with my educational supervisor, Professor Stephen L Atkin, and we answered ethical questions surrounding the study raised by a panel of 14 members from different background including doctors, nurses, acadaemics, lay people, and REC managers. The study was only conducted after receiving a favourable REC's decision. All study participants signed an informed consent form before participation.

2.3.3 Study participants.

Women with PCOS and controls were invited for a screening visit if they had a body mass index (BMI) between 30 – 45 kg/m^2, were between 18 – 45 years of age and were not taking the oral contraceptive pill or any medications that might influence study results including metformin. Women with PCOS were recruited from a secondary care out-patient clinic at Hull Royal Infirmary and through an advertisement in the local newspaper. PCOS was diagnosed according to the Rotterdam criteria (7). The diagnosis was confirmed if women had oligomenorrhoea

(defined as having fewer than 9 periods/year), clinical or biochemical hyperandrogenism, and features suggestive of PCOS in the ovaries on trans-vaginal ultrasound (TV USS) scan. Other endocrine disorders with similar presentation were excluded including hypothyroidism, hyperprolactinaemia, Cushing's disease, hypopituitarism, androgen producing tumours, and non-classic congenital adrenal hyperplasia (NC-CAH).

Control subjects were recruited through an advertisement in the local newspaper, requesting overweight females between 18 – 45 years of age, and underwent the same biochemical screening as the PCOS group to exclude any unknown endocrine disorder. Women with a history of clinical or biochemical hirsutism or menstrual irregularities were excluded. TV USS scan was not performed in the control group as the procedure was not clinically indicated and its suggestion was looked on unfavourably by the local ethics committee. Controls were screened at the follicular phase of the cycle. This was not possible for women with PCOS due to oligomenorrhoea. Women with alcohol intake of >14 units/week were excluded from the study. The screening visit included having an oral glucose tolerance test (OGTT) done. cIMT was performed at visit two, and endothelial function and markers of atherothrombosis were measured at visit three, in the fasting state. In some participants visit two and three were combined. All tests were performed under the same experimental conditions.

2.3.4 Anthropometric measures.

Anthropometric measurements were done by the same person for consistency. Body mass index (BMI), calculated as weight in kilograms divided by the square of height in meters. Waist circumference was defined as the widest circumference at the midpoint between the lateral iliac crest and the lowest rib margin at the end of normal expiration. Hips circumference was defined as the widest circumference over the femoral heads.

2.3.5 Biochemical markers.

Venous blood samples were collected in the morning of study visits after a minimum of 10 hours fast. Samples were separated by centrifugation at 2000g for

15 minutes at 4°C, and the aliquots stored at –80°C until batch analysis. Measurements were performed by a lab technician (A.A) who was blinded to study group of participants. High sensitivity C-reactive protein (hsCRP) was measured using Synchron systems CRPH reagent kit (Beckman-Coulter, UK) as per manufacturer's protocol. hCRP concentration measured has an analytical range of 0.2 – 80 mg/L in serum or plasma collected within 2 hours. Serum testosterone was measured by tandem mass spectrometry and sex hormone binding globulin (SHBG) by an immunometric assay with fluorescence detection on the DPC Immulite 2000 analyzer. Free androgen index (FAI) was calculated as the total testosterone x 100/SHBG. Serum insulin was assayed using a competitive chemiluminescent immunoassay performed on the manufacturer's DPC Immulite 2000 analyzer (Euro/DPC, Llanberis, UK). The analytical sensitivity of the insulin assay was 2µU/ml, the coefficient of variation was 6%, and there was no stated cross-reactivity with proinsulin. Fasting plasma glucose (FPG) was measured using a Synchron LX 20 analyzer (Beckman-Coulter) using the manufacturer's recommended protocol. The coefficient of variation for the assay was 1.2% at a mean glucose value of 5.3 mmol/L during the study period.

IR was measured using the Homeostasis Model Assessment (HOMA) as HOMA-IR= (FPG (mmol/L) X fasting insulin (iu/ml))/22.5 (273). Total cholesterol, triglycerides, and high-density lipoprotein (HDL) cholesterol levels were measured enzymatically using a Synchron LX20 analyzer (Beckman-Coulter, High Wycombe, UK). Low-density lipoprotein cholesterol (LDL) was calculated using the Friedewald equation [LDL (mmol/L)= total cholesterol – HDL – (0.45*triglycerides)] (274, 275).

2.3.6 Oxidative stress.

Oxidative stress is caused by excess production of reactive oxygen species (ROS), such as superoxide, hydrogen peroxide and hydroxyl radicals, which are not adequately inactivated by antioxidants and could result in cellular damage. ROS are able to react with DNA, proteins and lipids and these reactions and their products can induce a number of abnormal cellular responses including proliferation, prevention of cell division, DNA fragmentation, senescence, apoptosis or necrosis (276). Instead of ROS, which are difficult to measure (unstable and have short half-

lives), their stable metabolites or molecular products are more commonly measured such as lipid peroxidation end products or oxidised proteins (276, 277). Isoprostanes are stable end-products of arachidonic acid produced through a non-enzymatic process of lipid peroxidation, catalysed by oxygen free radicals on cell membranes and LDL particles (278). There are a large number of possible isoprostane end products, but 8-iso prostaglandin $F_{2\alpha}$ (8-iso $PGF_{2\alpha}$) has been widely investigated (279, 280). Isoprostanes, usually 8-iso $PGF_{2\alpha}$, can be measured either in urine or plasma (281, 282). Only the hydrolyzed forms of isoprostanes are present in urine and are more stable. Overnight urine samples were collected and aliquots stored at -20°C until batch analysis. Urinary isoprostane, 8-iso $PGF_{2\alpha}$, was measured by enzyme-linked immunosorbent assay (ELISA) using urinary isoprostane EIA kit (Oxford Biomedical Research, Oxford, USA) as described below by an operator who was blinded to study group of participants.

2.3.6.1 Enzyme-linked immunosorbent assay (ELISA).

Principle: In ELISA, an antigen must be immobilized to the assay plate by direct adsorption or by first attaching to a capture antibody to the plate surface. Detection of the antigen can then be performed using an enzyme-conjugated primary antibody (direct detection) or a matched set of unlabelled primary and conjugated secondary antibodies (indirect detection) (Figure 2.1). Detection is accomplished by assessing the conjugated enzyme activity via incubation with a substrate to produce a measureable product (usually colour). The test is highly dependent on specific antibody-antigen interaction.

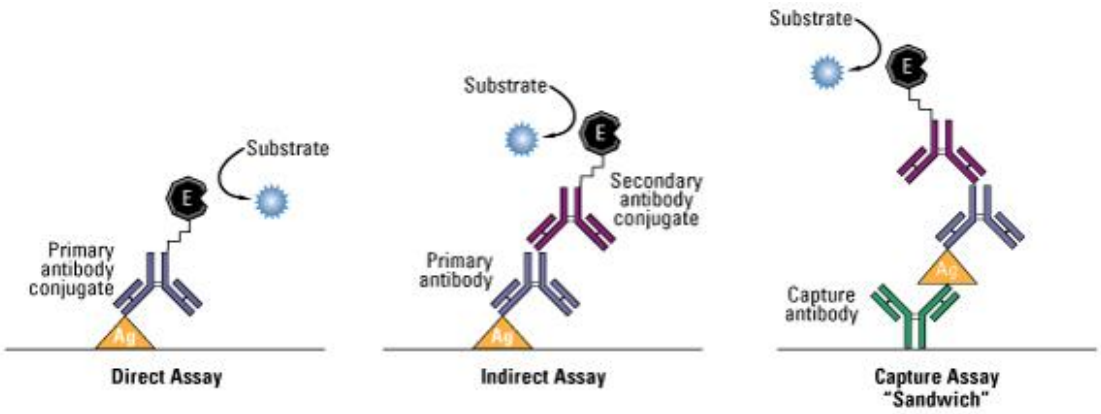


Figure 2.1 Direct vs. Indirect detection ELISA. The first step is for the antigen (Ag) to be immobilized to the assay plate either by direct adsorption or by attachment to a capture antibody on the plate surface. In direct Enzyme-linked immunosorbent assay (ELISA) assay the Ag is detected directly using an enzyme-conjugated primary antibody. In an indirect assay the Ag is detected first by an unlabelled primary antibody and then by an enzyme-conjugated secondary antibody. E, enzyme. Obtained from Douglas Hayworth (283).

Procedure: Urinary isoprostane EIA kit was supplied by Oxford Biomedical Research (Oxford, US). This assay is a competitive enzyme-linked immunoassay based on competition between 8-iso-PGF2α and 8-iso-PGF2α-horseradish peroxidase (HRP) for a polyclonal goat anti-8-iso-$PGF_{2\alpha}$ antibody (Figure 2.2).

Urine samples were diluted 1:6, as per manufacturer's protocol, in enhanced dilution buffer and 100µl added in duplicate to a 96-well plate coated with polyclonal anti-8-iso-$PGF_{2\alpha}$ antibody. An 8-iso-$PGF_{2\alpha}$ standard was diluted in enhanced dilution buffer to give concentrations of 0.05, 0.1, 1, 5, 10, 50, 100 pg/ml and 100µl added in duplicate to the 96-well plate. 100µl of 8-iso-$PGF_{2\alpha}$-HRP conjugate was added to each well and the plate was incubated at room temperature for 2 hours. A reagent blank (RB) was assessed by addition of 200µl enhanced dilution buffer and maximum binding (B0) was assessed by addition of 100µl buffer and 100µl of 8-iso-PGF2α-HRP conjugate. The plate was washed 3 times with 300µl of wash buffer before addition of 200µl of substrate (3, 3', 5, 5'-tetramethylbenzidine, TMB) to each well. The ELISA was developed for 40 minutes

before the addition of 50µl of 3M sulfuric acid to each well to stop the reaction and the plate read at 450nm in a Tecan Classic plate reader (Mannedorf, Germany).

Duplicate absorbance values were averaged and the reagent blank (RB) value was subtracted from all values. The %B/B0 was calculated by dividing the sample/standard by the B0 value and multiplying by 100. The standard %B/B0 was plotted against the log transformed concentration and a curve fitted by nonlinear regression (sigmoidal dose response curve) to derive the concentration of 8-iso-$PGF_{2\alpha}$ in each sample.

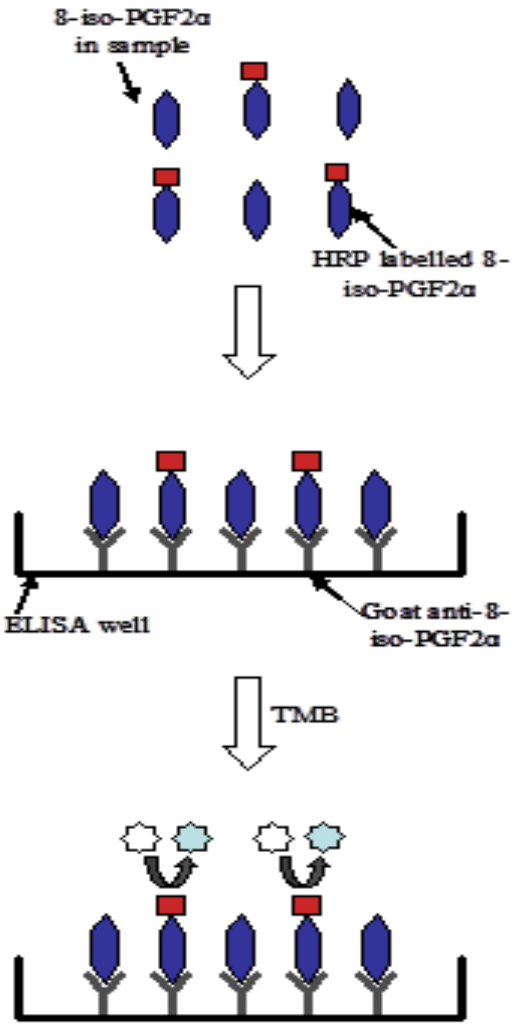


Figure 2.2 Diagram illustrating ELISA method for measurement of urinary 8-iso-$PGF_{2\alpha}$. In the first stage, 8-iso-$PGF_{2\alpha}$ (8-iso prostaglandin $F_{2\alpha}$) in the participant's urine sample and 8-iso-$PGF_{2\alpha}$-horseradish peroxidase (HRP), from the isoprostane

kit, compete to bind to a polyclonal goat anti-8-iso-$PGF_{2\alpha}$ antibody on the plate's surface. After several washing steps, a substrate (3, 3', 5, 5'-tetramethylbenzidine, TMB) is added which interacts with 8-iso-PGF2α-HRP to produce a colour. The denser the colour, the lower the concentration of 8-iso-$PGF_{2\alpha}$ in the urine sample.

2.3.7 CIMT measurements.

cIMT was defined as a double-line pattern visualized by ultrasound on both walls of the common carotid artery (CCA) in a longitudinal image. It is formed by two parallel lines, which consist of the leading edges of two anatomical boundaries: the lumen- intima and media-adventitia interfaces (131).

A Plaque was defined as a focal structure encroaching into the arterial lumen of at least 0.5 mm or 50% of the surrounding cIMT value, or demonstrating a thickness > 1.5 mm as measured from the intima-lumen interface to the media-adventitia interface (131) (Figure 2.3).

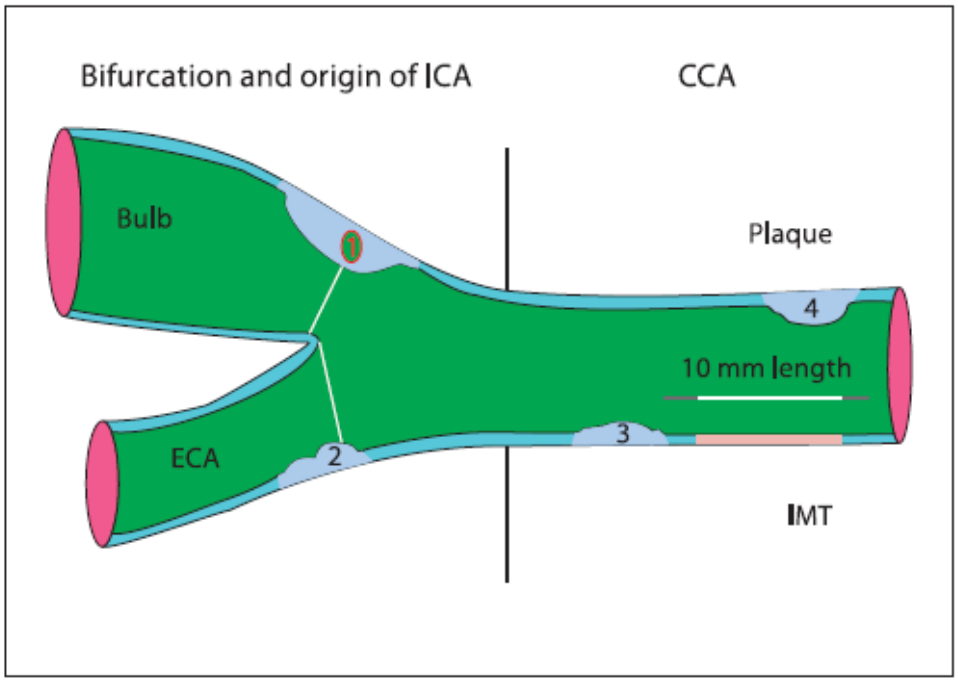


Figure 2.3 A representation of carotid tree, with plaque and IMT measurement according to Mannheim consensus. 1: thickness >1.5 mm; 2: lumen encroaching >0.5 mm; 3, 4: >50% of the surrounding IMT value. Obtained from Touboul et al. (131).

Procedure: A Toshiba Xario 15 scanner (Toshiba Medical Systems, Tokyo, Japan) equipped with 11-MHz linear imaging probe was used. I performed all the scans for consistency, after receiving the appropriate training. The cIMT measurements were performed in accordance with recommendations by Mannheim consensus, 2006 (131).

Participants were asked to lie in a supine position with the neck in the neutral position and in slight hyperextension. Image parameter settings were: depth of focus 20 – 30 mm, frame rate 24Hz, log gain compensation 60dB. Time gain compensation controls were adjusted to obtain optimal intraluminal artefacts. On average 10 images were taken in the anterolateral view from the right CCA at the end of diastole, from an area without local thickening, at a distance of 1 – 2 cm proximal to the origin of the bulb. Images were saved in DICOM format with minimal compression (Figure 2.4).

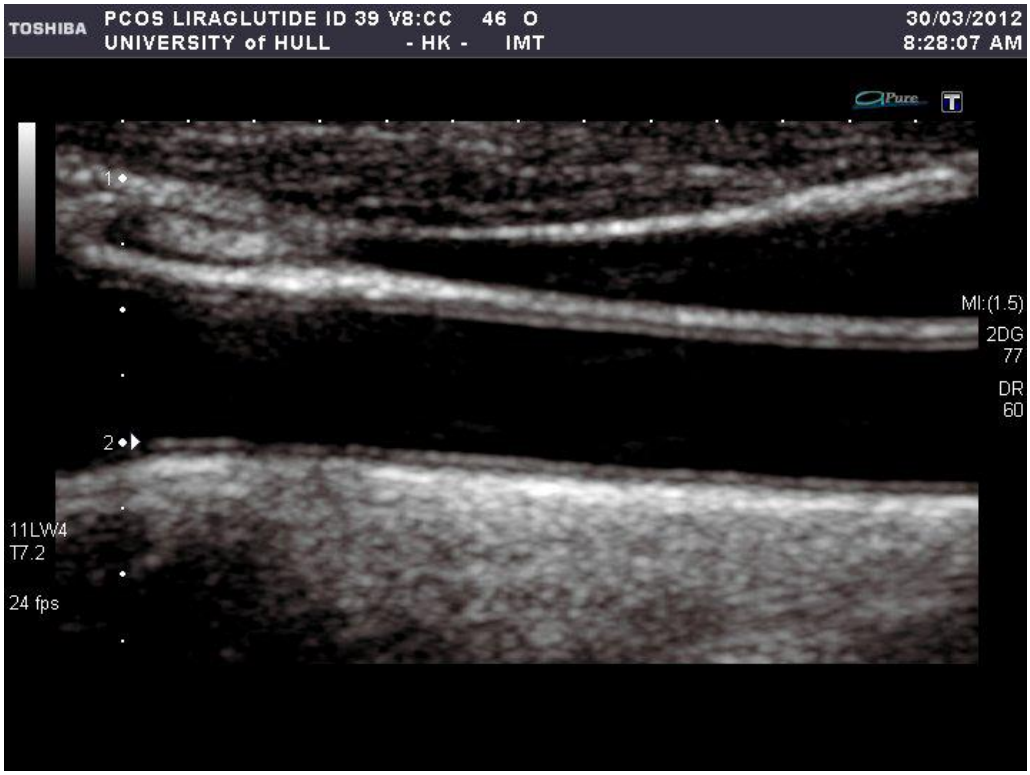


Figure 2.4 An example of an image taken from the common carotid artery for cIMT measurement. The image was taken from the right common carotid artery. The focus of the image was the far wall of the artery (arrow). At the far left of the image we can see the origin of the bulb (dotted vertical line). cIMT measurement

was taken at a distance of 1 – 2 cm from the origin of the bulb across a 1 cm segment.

The average cIMT across 1 cm segments from the far wall of CCA was measured by an independent operator who was blinded to the study group of participants, using automated software, SonoCalc IMT 4.1 (SonoSite Ltd, UK).

Reproducibility of cIMT measurements was assessed using a Bland Altman plot in 18 participants who underwent 2 ultrasound examinations within a 4 week period (Figure 2.5), mean difference=-0.002 (95% limits of agreement=-0.055, 0.059); the intraclass correlation coefficient was very high (ICC=0.95, 95% CI=0.90, 0.99).

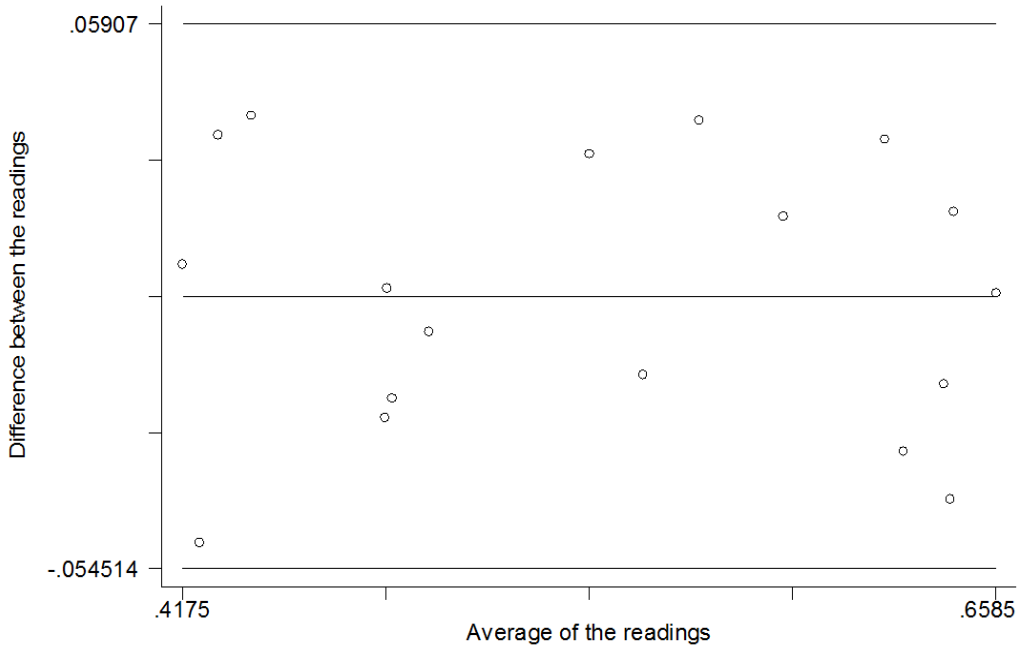


Figure 2.5 Bland Altman plot for reproducibility of cIMT measurements. To assess reproducibility, 18 participants underwent two cIMT measurements within two weeks. The middle horizontal line represents the mean difference between the two measurements =-0.002. As the mean difference is close to zero, there is high agreement between the two measurements. The upper and lower horizontal lines represent the 95% confidence interval for the difference (i.e mean difference ± 1.96 standard deviation of the difference).

2.3.8 Platelet function.

Platelets play a key role in atherosclerosis (153); despite this, there are only a few clinical tests to assess platelet function and these tests are not without limitations (284). For example, although platelet-aggregation studies are commonly used, they are subject to artifactual platelet activation (during sample preparation) and 'normal' responses differ between people without disease (285). Another clinical test that was commonly used to assess platelet function is bleeding time, but its reproducibility and clinical relevance are questionable and the test could be influenced by factors other than platelets, including anaemia and coagulopathies (285). Furthermore, while activated platelets produce thromboxane A_2 (T_XA_2) and higher levels of plasma or urinary T_XA_2 metabolites may determine platelet activation (286, 287); this method cannot measure the extent of activation of individual platelets or detect distinct subpopulations of platelets (284).

A more recent method to assess platelet function is flow cytometry in which a large number of individual cells can be examined (flow cytometry is explained in detail in chapter three, paragraph 3.3.6). Whole blood flow cytometry has the advantage of examining individual platelets in their own environment and with minimal sample manipulation (288). Platelet function can be assessed by examining the expression of activation-dependent antigens on the platelet surface. P-selectin (also known as CD62P) is a component of the *α* granule membrane of resting platelets and is only expressed on the platelet surface membrane after *α* granule secretion (i.e. platelet activation) (289). P-selectin mediates adhesion of activated platelets to neutrophils and monocytes which may play an important role in the pathogenesis of inflammation and thrombosis (290). When platelets are activated, the GPIIb-IIIa complex on the platelet surface undergoes a conformational change resulting in exposure of the fibrinogen binding site and subsequent fibrinogen binding, an essential step in platelet aggregation and thrombus formation (291). Therefore the binding of fibrinogen to the platelet surface can be used as a marker of platelet activation.

Measuring these markers by whole blood flow cytometry in unstimulated samples, that is, in the absence of an added exogenous platelet agonist provides information

on the activation state of circulating platelets *in vivo*. The inclusion of an exogenous agonist in the assay enables analysis of the reactivity of circulating platelets *in vitro* (284). Surface expression of P-selectin and fibrinogen binding correlates with subsequent cardiac risk (189) and has been found to be increased in people with cerebrovascular disease (187), diabetes and obesity (188).

Procedure: Platelet function was analysed in whole blood by flow cytometry according to the method of Goodall et al. (292). Venous blood was collected by me without stasis from the antecubital vein into a syringe with 3.8% sodium citrate using a 21 gauge butterfly needle by standard venepuncture technique. The first 2ml of blood were discarded to avoid artifactual platelet activation. Within 5 minutes of blood collection, 5µL of citrated blood was diluted in 50µL of modified Tyrodes buffer (150mM NaCl, 5mM HEPES [N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid], 0.55mM NaH_2PO_4, 7mM $NaHCO_3$, 2.7mM KCl, 0.5mM $MgCl_2$, 5.6mM glucose, pH 7.4) and mixed with 2µL of fluorescein isothiocyanate-conjugated (FITC) anti-CD42b monoclonal antibody (BD Biosciences Oxford, UK), FITC anti-fibrinogen polyclonal antibody (DakoCytomation, Stockport, UK); phycoerythrin-conjugated (PE) anti-P-selectin monoclonal antibody or IgG isotype control (BD Biosciences Pharmingen, San Jose). Samples were fixed with 0.2% paraformaldehyde and analysed within 3 hours of fixation by flow cytometry. Platelet population was identified by forward and side scatter characteristics and confirmed by the expression of the platelet specific surface marker CD42b. Fibrinogen binding and P-selectin expression were calculated from 10,000 platelets 'events'.

To study the sensitivity of platelets to activation, diluted blood was supplemented with ADP and samples were fixed with 500µl of 0.2% paraformaldehyde and processed by flow cytometry (292). Fixing samples at 20 minutes provided the maximum expression of P-selectin and fibrinogen binding in response to stimulation with ADP (Figure 2.6). Using increasing doses of ADP 0.1 - 10µM permitted the studying of platelet function in response to mild – maximum stimulation (Figure 2.6).

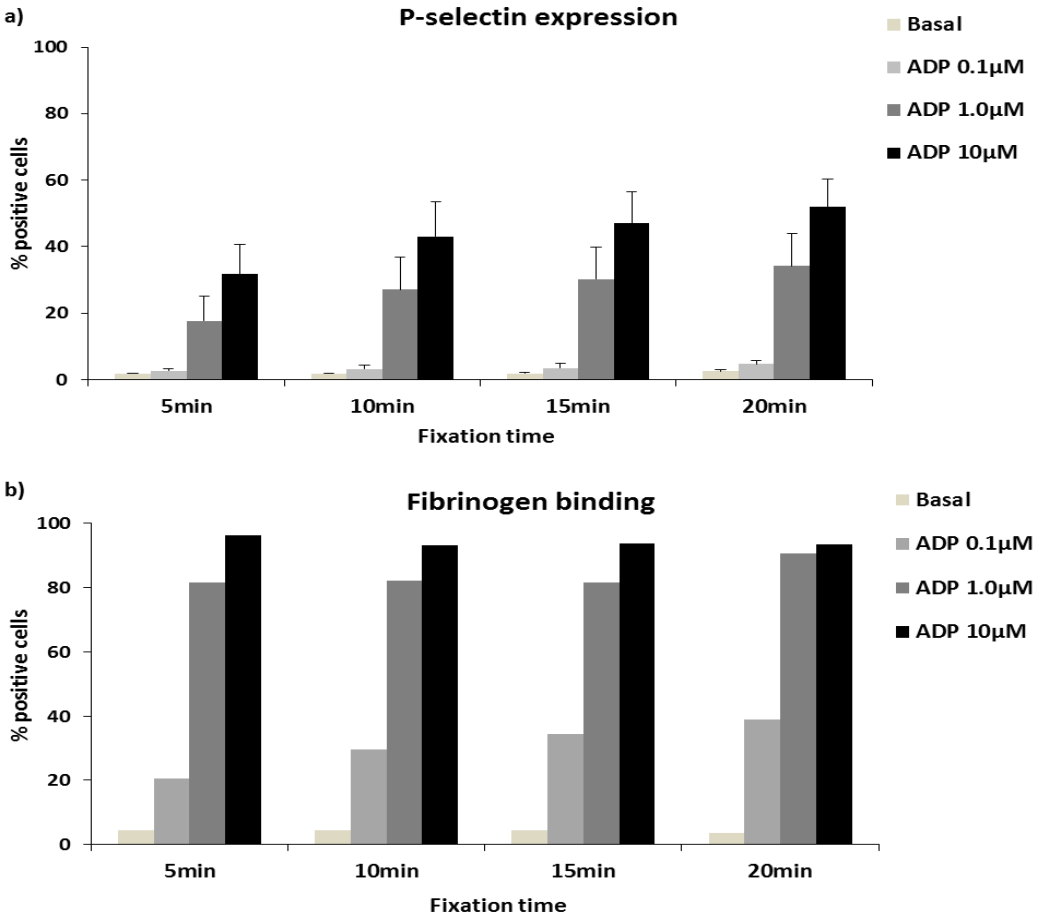


Figure 2.6 The effects of different fixation times on the expression P-selectin and fibrinogen binding on platelet surface. Whole blood samples were collected from healthy volunteers. Samples were incubated with ADP 0.1 – 1µM before fixation with 500µl of 0.2% formaldehyde at 5 – 20 minutes. Samples were analysed by flow cytometry. **Fig 2.6a** platelet surface expression of P-selectin, N= 3 independent experiments, data presented as mean ±standard error of the mean (SEM). **Fig 2.6b** platelet fibrinogen binding, N= 1 experiment. Maximum expression of P-selectin and fibrinogen binding on platelet surface was noted when samples were fixed at 20 minutes.

In some experiments sample were incubated with prostacyclin (PGI_2) (Cayman Chemical, USA) for 90 seconds prior to adding ADP. Incubation times, agonists and inhibitors concentrations were optimised to achieve maximum binding between antibody and receptor. The best results, allowing to test mild – maximum platelet inhibition with PGI_2, were found when samples were incubated with PGI_2 1 – 100nM for 90 seconds before stimulation with either ADP 10µM and fixing at 20

minutes for P-selectin expression (Figure 2.7a), or ADP 1µM and fixing at 5 minutes for fibrinogen binding (Figure 2.7b).

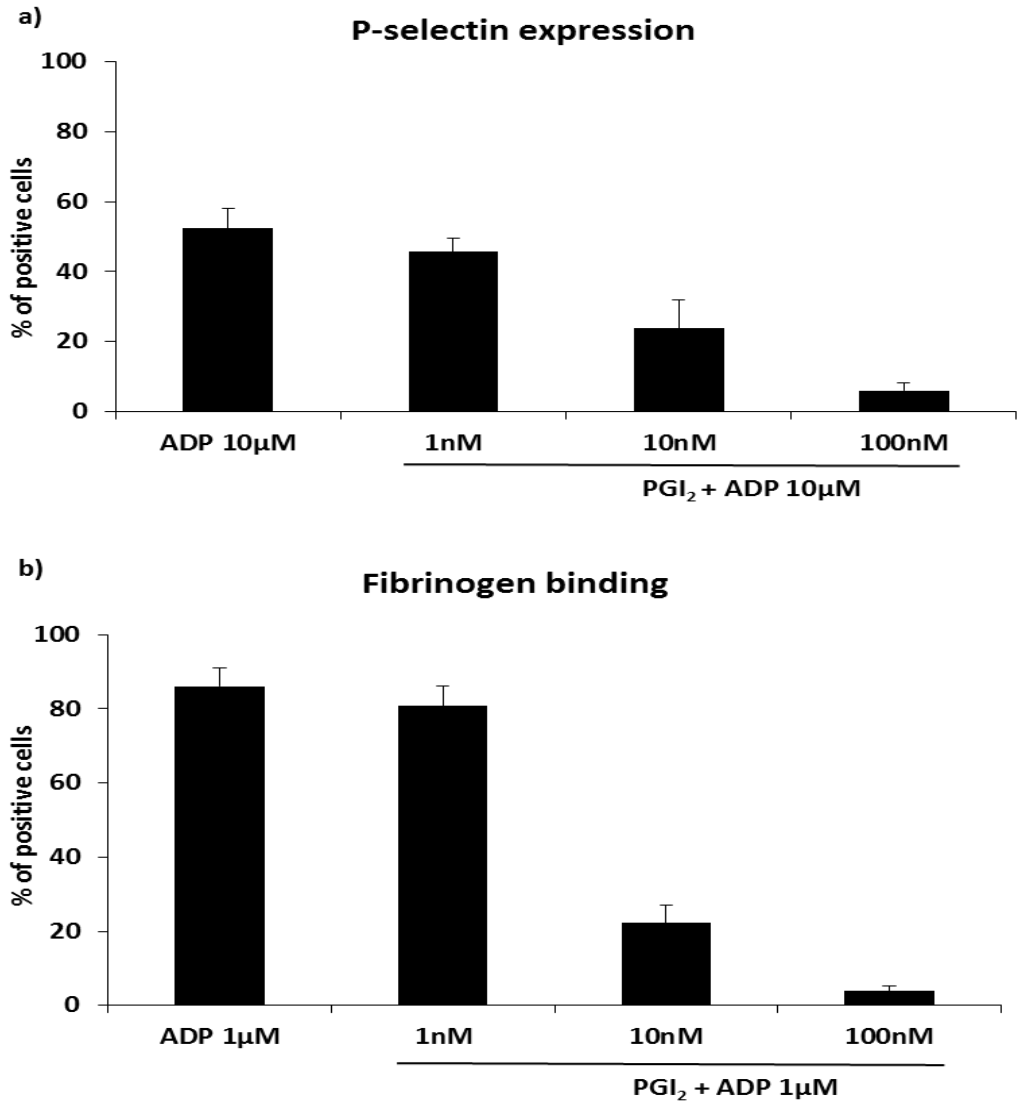


Figure 2.7 Platelets response to the inhibitory effects of PGI_2. Fig 2.7a For P-selectin expression, samples were incubated with prostacyclin (PGI_2) 1 – 100nM for 90 seconds before stimulation with adenosine diphosphate (ADP) 10µM and fixing at 20 minutes with 500µl of paraformaldehyde. **Fig 2.7b** For fibrinogen binding, samples were incubated with PGI_2 1 – 100nM for 90 seconds before stimulation with ADP 1µM and fixation at 5 minutes. N=3 independent experiments. As PGI_2 dose increases, more inhibition in the expression of P-selectin and fibrinogen binding is noted. Data presented as mean ±standard error of the mean (SEM).

The expression of P-selectin and fibrinogen binding on the platelet surface remained stable when samples were analysed 3 hours after fixation (Figure 2.8). Data were presented as percentage of platelets expressing P-selectin and fibrinogen binding as this was found to correlate with subsequent cardiac risk (189).

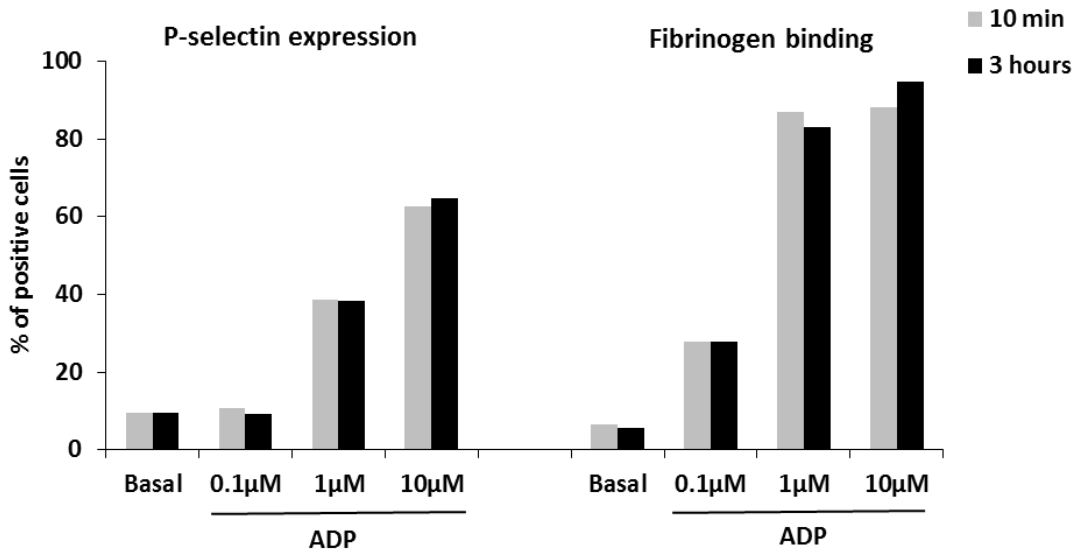


Figure 2.8 The effects of delay in sample analysis after fixation on P-selectin and fibrinogen binding expression. Whole blood samples were incubated with a vehicle (basal) or adenosine diphosphate (ADP) 0.1 – 10µM before fixation at 20 minutes with 500µl of 0.2% paraformaldehyde. Samples were analysed by flow cytometry at 10 minutes and 3 hours after fixation. The expression of P-selectin and fibrinogen binding was similar when samples were analysed immediately or after 3 hours of fixation. N=1 experiment.

2.3.9 Endothelial function.

The endothelial cells control vascular tone and produce different activators and inhibitors of platelet function and blood clotting (Figure 2.9) (153). When a group of researchers removed the endothelium from the wall of a blood vessel and infused acytelcoline, the vessel paradoxically constricted, in comparison to dilation in the vessel with healthy endothelium (293). NO is produced by the endothelium and is an important vasodilator. The ability of blood vessels to dilate in response to a stimulus reflects endothelium health and is a measure of endothelial function. Coronary vessels with atherosclerotic plaques dilate less in response to stimulants compared to vessels from healthy people. Similar findings were noted in patients

with CV disease (294). Examining the endothelial function of the coronary arteries by measuring coronary epicardial vasoreactivity is widely considered as the gold standard as it examines the vessels which are clinically important (295). However the technique is invasive, risky and requires operator's expertise. Other methods for measuring endothelial function in the peripheral vessels, including flow mediated dilatation (FMD) and Endo-PAT200, are less invasive and correlate well with coronary endothelial function.

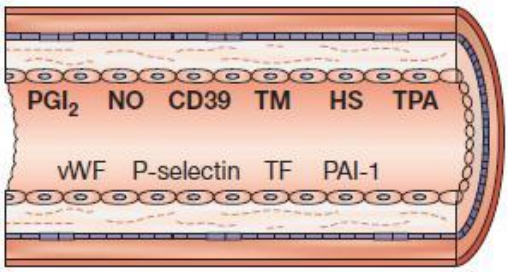


Figure 2.9 Balance between antithrombotic and prothrombotic molecules synthesised by endothelial cells. Prostacyclin (PGI_2) and nitric oxide (NO) inhibit platelet activation by increasing the intracellular levels of cAMP and cGMP; CD39, an 'ectoADPase' that degrades ADP, and thrombomodulin (TM), which changes the substrate specificity of thrombin, limit platelet stimulation by these agonists; heparan sulfate (HS) participates in the inactivation of thrombin; and tissue plasminogen activator (TPA) leads to the generation of plasmin that degrades fibrin. Among the thrombogenic substances, vWF and P-selectin mediate platelet and leukocyte adhesion; tissue factor (TF) initiates the coagulation cascade that leads to fibrin generation; and plasminogen activator inhibitor 1 (PAI-1) inhibits the formation of plasmin and opposes thrombus dissolution. Obtained from Ruggeri et al. (153).

When the blood supply in a limb is interrupted for a limited period of time and then released, the vessel wall dilates, this is known as reactive hyperemia. The most widely used technique to assess endothelial function is FMD of the arm arteries (295). In FMD the brachial artery diameter is measured by ultrasound and the change in diameter in response to 5 minutes of blood supply occlusion to the arm is

recorded. FMD has been found to correlate with coronary endothelial dysfunction and is reduced in patients with coronary vascular disease (296). FMD measures changes in the endothelial function which are mainly dependent on NO release from the endothelium but other factors may affect the results such as, exercise, intercurrent illness, temperature, and food/beverages that contain alcohol or caffeine or are rich in polyphenols (cocoa, tea, fruit juices) (295). In addition, FMD requires operator expertise and to compare results between studies standardisation of the technique is needed (295).

2.3.9.1 Endo-PAT2000.

Endo-PAT is a validated method for measuring endothelial function at the peripheral vessels (297). Endo-PAT2000 measures the change in pulse wave amplitude (PAT) at the finger tips in response to reactive hyperemia. It is not invasive, simple to perform (doesn't require operator's expertise), reproducible (298) and lower scores have been associated with increased CV risk in clinical studies (299). Despite being easy to use, Endo-PAT is an expensive test as probes are not reusable and are expensive to buy. In addition, changes measured by Endo-PAT are only partly dependent on NO and are influenced by non-endothelial factors, like autonomic function (295). Interestingly in the Framignham study Endo-PAT and FMD were poorly correlated which suggests that they might be measuring two different things (300). It is proposed that FMD measures endothelial function at the macrovascular level while Endo-PAT measures the microvascualr circulation.

Procedure: Endo-PAT2000 was obtained from Itamar Medical Ltd, Israel. All tests were performed in the fasting state, in a quiet and temperature controlled room (23°C), with the participant relaxed in a semi-recumbent position. All tests were performed by the same operator. Special probes were placed on the index finger of each arm to measure PAT. The probes were inflated to apply a pressure on the fingertips of around 70 mmHg to prevent venous congestion. The left arm was used as the study arm and the right arm was used as control. A blood pressure cuff was applied to the study arm at the level of the brachial artery. PAT was recorded in both arms for the duration of the test. After 5 minutes of rest, baseline, the blood pressure cuff was inflated to 220 – 230 mmHg to stop the blood supply in the study

arm for 5 minutes, and then deflated to release the blood supply for a further 5 minutes (Figure 2.10). A reactive hyperemic index (RHI) is a measure of the change in PAT in the study arm after occlusion compared to baseline and adjusted for changes in the control arm. RHI is calculated as the average PAT at 60 – 120 seconds after the release of the occlusion, divided by the average PAT over 3.5 minutes at baseline, and corrected for the change in PAT in the control arm over the same period.

a)

b)

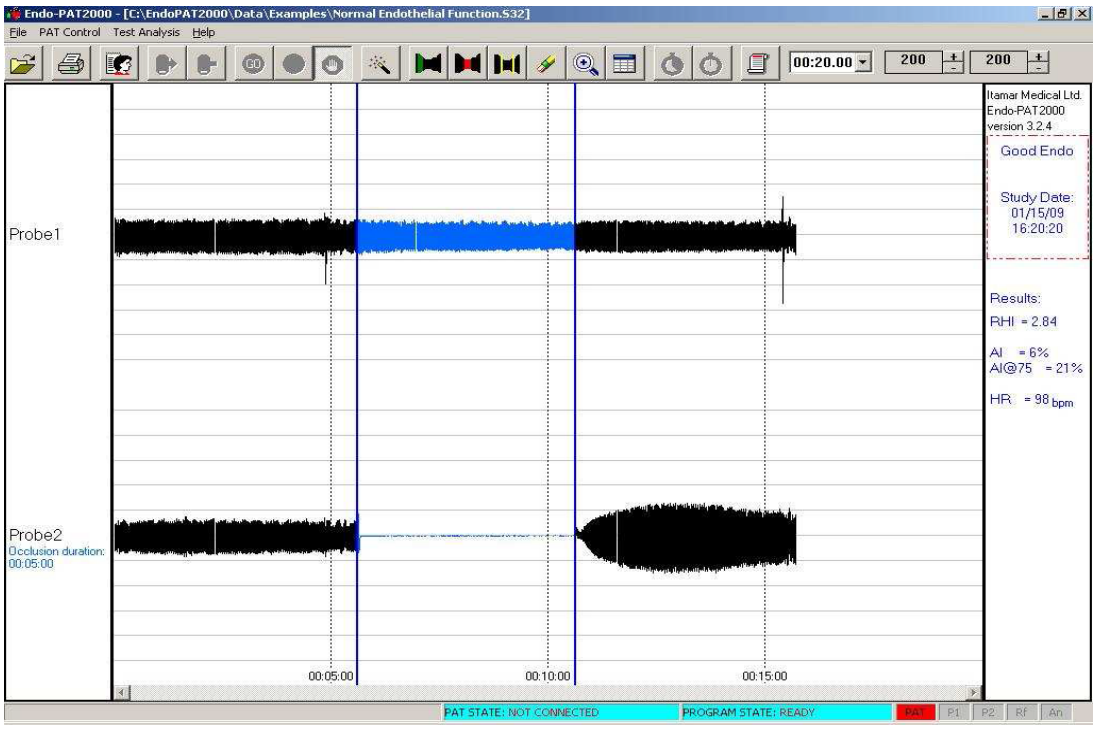


Figure 2.10 A representation of Endo-PAT2000. Fig 2.10a The Endo-PAT2000 device. **Fig 2.10b** Study output: the upper trace represents the signal obtained from

probe 1 (control arm), and the lower trace represents the signal obtained from probe 2 (study arm). The two vertical lines, in blue, highlight when the blood pressure was increased in the study arm to stop the blood supply between minutes 5 – 10. While no signal could be captured from the study arm during the occlusion period, the signal from the control arm was continuous. After 5 minutes of occlusion the blood pressure cuff was released and the signal was recorded from both arms for 5 minutes. The duration of the test is 15 minutes. The increase in blood flow in the study arm after occlusion resulted in a signal with a higher amplitude compared to baseline. The difference between these two signals (after vs. before occlusion) is adjusted for non-specific changes recoded from the control arm and is presented as reactive hyperemic index (RHI). In this example, RHI was 2.84 as presented in the right panel of study output. Obtained from Endo-PAT2000 user manual (301).

Interpretation of results: Bonetti et al. (297) reported that a measurement of RHI lower than the cut-off value of 1.67 provides a sensitivity of 82% and a specificity of 77% for diagnosis of coronary endothelial dysfunction. RHI has been found to be reduced in people with coronary disease, obesity, and diabetes but interestingly increased with age (267). It also correlates with coronary endothelial function and was found to predict future cardiovascular risk (299). Endo-PAT reproducibility was found to be good, ICC=0.74, and coefficient of variation 12 ±2.2% (298).

2.3.9.2 Serum endothelial adhesion markers.

Atherosclerosis usually occurs over a period of many years. The early lesions of atherosclerosis are characterized by the recruitment of leukocytes, especially monocytes, to the artery wall. The pathogenesis is multifactorial and is believed to start with the accumulation of lipoproteins in the subendothelium (182). These lipoproteins undergo oxidative modification and the accumulation of minimally oxidised LDL stimulates the overlying endothelial cells to produce a number of proinflammatory molecules, including adhesion molecules and growth factors which promote leukocyte recruitment to the site of the lesion. The adhesion molecules including P-selectin, E-selectin, cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) bind to carbohydrate ligands on

leukocytes and facilitate leukocyte adhesion to the endothelial surface (182). After migration, some monocytes ingest lipids and become foam cells, initiating a complex chain of events ending in atherosclerotic lesion development (182).

The important role of adhesion molecules in atherosclerosis was highlighted in animal studies. P-selectin-, E-selectin- or ICAM-deficient mice were found to have less atherosclerotic lesions compared to controls (302); while blocking the interaction between very late antigen-4, expressed on monocytes, and VCAM-1 was found to reduce atherosclerotic plaques in wild-type mice (303). The appearance of soluble cell adhesion molecules in the circulation is thought to result from the release of these molecules by activated endothelial cells reflecting their increased expression on the cell surface (304). Higher levels of serum endothelial adhesion molecules have been reported in a number of pathological conditions including diabetes, hypertension, dyslipidaemia and atherosclerosis (305-307).

Procedure: The serum concentrations of endothelial adhesion markers sICAM-1, sVCAM-1, sE-selectin and sP-selectin were measured by commercially available quantitative ELISA (R&D Systems, USA) according to the manufacturer's instructions. Samples were assayed in duplicate by an operator blinded to the study group of participants.

2.3.10 Clot structure analysis.

Alterations in clot structure/function have been reported in people with CV disease. Clots formed from the plasma of patients with myocardial infarction were stiffer and denser (comprising thinner, shorter, and more numerous fibrin fibers), and lysed more slowly than healthy individuals (270, 308). Altered clot structure and decreased permeability was also noted in healthy first-degree relatives of individuals with CV disease, suggesting abnormalities in clot structure and function predate the development of disease (309, 310).

Procedure: Clot structure analysis was measured using turbidimetric analysis as previously described (310). In the turbidimetric clotting assay dilute plasma and low thrombin concentration were used to prolong the lag time, which represents the time at which sufficient protofibrils have formed to enable lateral aggregation,

enabling analysis in 96-well plates, as follows: 25 µL of citrated plasma (in duplicate) was added to 75 µL assay buffer (0.05 mol/L Tris-HCl, 0.1 mol/L NaCl, pH 7.4), 50 µL of activation mix (final concentrations: 0.03 U/mL thrombin [Calbiochem], and 7.5 mmol/L calcium in assay buffer) was added to each column of the 96-well plate using a multichannel pipette at 10 sec intervals (the time of addition of activation mix was recorded to enable plate reader times to be adjusted to the start of clot initiation). Plates were shaken and the wells optical densities were read at 340 nm every 12 sec for 1 hour in a BIO-TEK ELx-808 microplate reader.

The turbidimetric lysis assay was carried out as above with the addition of 12.5 ng of tissue plasminogen activator (tPA) (Technoclone) to the 75 µL assay buffer (83 ng/mL final concentration) before addition of activation mix; this concentration of tPA gave complete lysis of pooled normal plasma within 1 hour. Plates were read at 340 nm every 12 sec for 1 hour and subsequently every 2 minutes for up to 9 hours.

The clot parameters assessed (Figure 2.11) included, lag phase (sec) which represents the time point at which an exponential increase in absorbance occurred; Maximum Absorbance (MA), a measure of fibrin network density and fibre thickness and was taken as the highest absorbance value adjusted for lag phase absorbance; clot formation time was calculated as the time from initiation of clot formation to the time of maximum absorbance; Lysis Time (LT) from full clot formation to 50% lysis, representing fibrinolysis potential; and Lysis Area (LA), area under the curve, representing a complex measure of clot formation, density and lysis potential. Higher MA of plasma clots, longer LT and larger LA are associated increased CV risk (311).

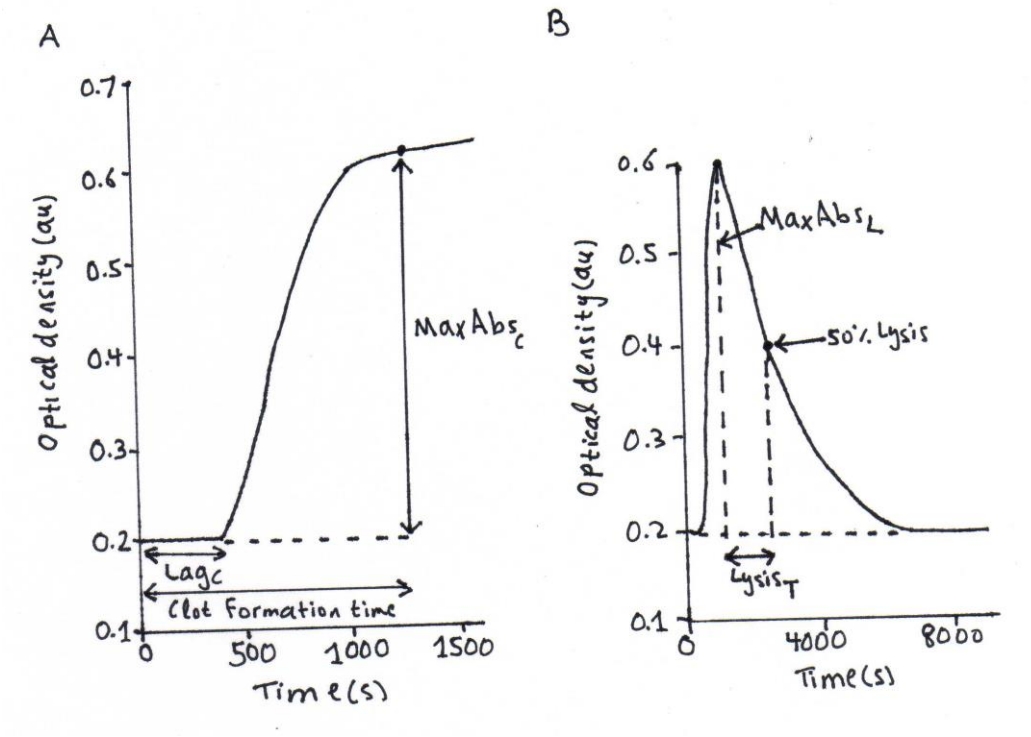


Figure 2.11. Illustration of turbidimetric clotting and lysis variables. Fig 2.11A, Turbidimetric clotting assay: lag time (LagC); maximum absorbance (MaxAbsC). **Fig 2.11B**, Turbidimetric lysis assay: maximum absorbance (MaxAbsL); lysis time from full clot formation to 50% lysis (LysisT); arbitrary units (au). Adapted from Carter et al. (310).

In a study by Carter et al. (310) including 537 subjects, fibrinogen contributed significantly to variance in all of the turbidimetric variables, accounting for 40.9% of variance in maximum absorbance (MA), 22.9% of variance in lysis area (LA), and between 1.5% and 10% of variance in other variables. Plasminogen activator inhibitor-1 (PAI-1) contributed to between 7% and 13% of variance in lysis variables (310).

2.3.11 Risk of diabetes, and metabolic syndrome.

All participants had an OGTT (312) at the screening visit. During an OGTT, plasma glucose was measured in the morning after a minimum of 10-hour fast and 2-hour after ingesting a drink containing 75 grams of glucose. The World Health Organisation defines: impaired fasting glucose (IFG) as FPG between ≥6.1 and

6.9mmol/L; impaired glucose tolerance (IGT) as 2-hour plasma glucose between ≥7.8 and <11.1mmol/L; and diabetes as FPG ≥7.0mmol/L or 2-hour plasma glucose ≥11.1mmol/L (312). Impaired glucose regulation (IGR) was defined as having IFG, and/or IGT on OGTT.

The metabolic syndrome was defined according to the International Diabetes Federation (IDF) criteria 2006 (313) as a waist circumference more than 80 cm and two of: triglycerides >1.7 mmol/L or specific treatment for this lipid abnormality; HDL < 1.29 mmol/L or specific treatment for this lipid abnormality; systolic BP ≥130 mmHg or diastolic BP ≥85 mmHg or treatment for previously diagnosed hypertension; raised FPG ≥ 5.6 mmol/L, or a previously diagnosed T2DM.

2.3.12 Power calculations and statistical analysis.

For cIMT measurements power was based on a standard deviation (SD) of 0.09mm (126). A sample size of 16 participants per group has 80% power, 5% significance (two-tailed) to detect one standard deviation score 'large effect size' for cIMT between cases and controls.

The sample size for platelet function was calculated using the Mann-Whitney U test for continuous data (314). The 'effect size' is taken from two papers (Table 2.1) (187, 315). A sample size of 18–20 per group allowed us to detect an effect size of 1.12 (or larger) with 80% power, 20% attrition and 5% significance (two-tailed).

There are no studies comparing platelet function between obese PCOS women and matched controls using flow cytometry. A paper by Rajendran et al. (125) found a significant difference in platelet aggregation between obese PCOS and lean controls with 12 patients/group. This gives us confidence in our earlier calculations.

Power (%)	Effect size	P (probability control<PCOS)	n/group	n/group (20% attrition)
80	1.25*	0.188	14	18
90	1.25*	0.188	18	23
80	1.12**	0.214	16	20
90	1.12**	0.214	22	28

Table 2.1 Power calculations for platelet function. P is the probability that an observation in the controls will be less than an observation in PCOS. The closer this probability is to 0.5, the larger the sample size. The null hypothesis is that the P=0.5 (medians equal). The numbers are for two groups of equal size while attrition is assumed non-differential. Calculations are subject to rounding errors. *From Pasupathy et al (2005).(315) ** From Yamazaki et al (2001).(187). Significance 5%, two-tailed.

Data were summarised by the mean and SD (continuous data) or by percentages (categorical data). Data were checked for normality using Kolmogorov-Smirnov test. Between groups analysis was performed using the independent t-test for continuous data (or Mann-Whitney U test for non-normally distributed data). Frequency distributions' were analysed using Chi-square test. Correlations between variables were evaluated by Pearson's coefficient (or Spearman's coefficient for non-normally distributed data).

Reproducibility for cIMT measurements was assessed using Bland Altman plot (316), and ICC (317). Statistical analysis was performed using the PASW statistics 18 package (SPSS Inc., Chicago, USA). A two tailed P value of <0.05 was considered statistically significant.

2.4 Results.

2.4.1 Demographics.

Fifty one women were screened, forty recruited (21 PCOS, 19 normal controls). Eleven women, from both groups, were excluded as they had; idiopathic hirsutism (five), hypothyroidism (one), BMI <30kg/m2 (one), no TV USS scan was done (two), ovaries not visible on TV USS (one) and oligomenorrhoea without PCOS (one). Out of the 21 women with PCOS, 12 (57%) fulfilled all three Rotterdam criteria, 9 (43%) fulfilled two criteria, oligomenorrhoea and hirsutism but no polycystic ovaries (PCO) on ultrasound. Study participants' demographics and baseline characteristics are summarised in Table 2.2.

	PCOS (n=21)	Controls (n=19)
Age (yr)	32.8 ± 7.2	33.5 ± 6.7
Weight (kg)	100.9 ± 16.7	99.3 ± 14.7
BMI (kg/m^2)	37.6 ± 4.9	36.1 ± 4.5
Waist (cm)	112.3 ± 12.6	111.5 ± 9.4
WHR	0.91 ± 0.07	0.91 ± 0.04
Systolic BP (mmHg)	122 ± 13.0	124 ± 16.1
Diastolic BP (mmHg)	76.2 ± 7.8	77.9 ± 8.7
Current Smokers	3 (5 pack-year)	3 (6 pack-year)

Table 2.2 Demographics and baseline characteristics for the PCOS and control groups. BMI, body mass index; WHR, wait to hip ratio; BP, blood pressure; pack-year, is a way to measure the amount a person has smoked over a long period of time; it is calculated by multiplying the number of packs of cigarettes smoked per day by the number of years the person has smoked (318). Data are presented as mean ±SD. All P values were >0.05.

2.4.2 Biochemical markers.

Women with PCOS had significantly higher FAI, and testosterone levels. Other markers did not differ significantly between the two groups including HOMA-IR, hsCRP, and fasting lipid profile; data are summarized in Table 2.3.

	PCOS (n=21)	Controls (n=19)	P
Testosterone (0 – 1.9 nmol/L)	1.2 ±0.3	0.9 ±0.3	0.01
FAI (0 – 4)	4.3 ±1.9	2.7 ±1.3	0.02
SHBG (35 – 100 nmol/L)	33.5 ±14.2	36.4 ±13.9	0.40
HOMA-IR	5.1 ±2.6	3.5 ±1.3	0.03
Insulin (iu/ml)	10.9 ±6.8	7.6 ±4.5	0.11
Plasma glucose (mmol/L)			
Fasting	5.1 ±0.6	4.9 ±0.4	0.39
2h after glucose challenge	6.3 ±2.0	5.7 ±1.5	0.31
hsCRP (0 – 8 mg/L)	6.2 ±8.9	5.1 ±3.4	0.35
Uric acid (0.15 – 0.38 mmol/L)	0.3 ±0.05	0.3 ±0.03	0.27
Total cholesterol (mmol/L)	5.0 ±0.8	5.1 ±1.0	0.82
LDL (mmol/L)	3.2 ±0.6	3.3 ±0.9	0.66
HDL (mmol/L)	1.2 ±0.2	1.3 ±0.2	0.31
Triglycerides (mmol/L)	1.4 ±0.7	1.4 ±0.7	0.89
Chol/HDL ratio	4.4 ±0.9	4.1 ±0.8	0.66

Table 2.3 Biochemical markers for the PCOS and control groups. FAI, free androgen index; SHBG, sex hormone binding globulin; HOMA-IR, homeostatic model assessment-insulin resistance; hsCRP, high sensitivity C-reactive protein.

2.4.3 CIMT measurements.

CIMT data were available on all study participants. There was no significant difference in average mean or maximum cIMT measurement between the two groups. Average cIMT (mm) was 0.5 ±0.05 vs. 0.48 ±0.06 (P=0.36), and maximum cIMT (mm) was 0.57 ±0.07 vs. 0.55 ±0.07 (P=0.30) for the PCOS and control groups, respectively.

2.4.4 Platelet function.

Platelet function data were available on 17 women with PCOS and 17 controls, with 6 fewer participants than for cIMT measurement as a consequence of 4 participants being lost to follow up after the initial cIMT and difficulty to obtain a clean venepuncture in 2 participants.

There was no significant difference between the two groups in platelets' surface expression of P-selectin or fibrinogen binding at basal conditions (unstimulated) or

in response to stimulation with ADP (0.1–10µM) (Table 2.4). Platelets' sensitivity to the endogenous platelet inhibitor PGI_2 (1–100nM) was also tested, and the inhibition of ADP induced platelet activation by PGI_2 was consistent between both groups (Figure 2.12).

	P-selectin			Fibrinogen binding		
	PCOS (n=17)	**Controls (n=17)**	**P**	**PCOS (n=17)**	**Controls (n=17)**	**P**
Basal	0.52 ±0.3	0.43 ±0.23	0.40	0.97 ±0.4	0.83 ±0.3	0.48
ADP 0.1µM	3.1 ±2.9	3.6 ±3.8	0.56	9.7 ±10.0	7.7 ±7.1	0.57
ADP 1µM	40.2 ±14.5	44.0 ±13.5	0.33	55.9 ±18.4	55.8 ±19.0	0.90
ADP 10µM	61.6 ±13.6	66.9 ±13.1	0.27	73.7 ±14.6	76.2 ±11.7	0.78

Table 2.4 Baseline platelet activation and response to ADP. Platelet surface expression of P-selectin and fibrinogen binding (% of positive cells) for the PCOS and control groups at baseline and in response to stimulation with ADP 0.1, 1, and 10µM. Samples were fixed at 20 minutes with 0.2% paraformaldehyde. Data are presented as mean ±SD.

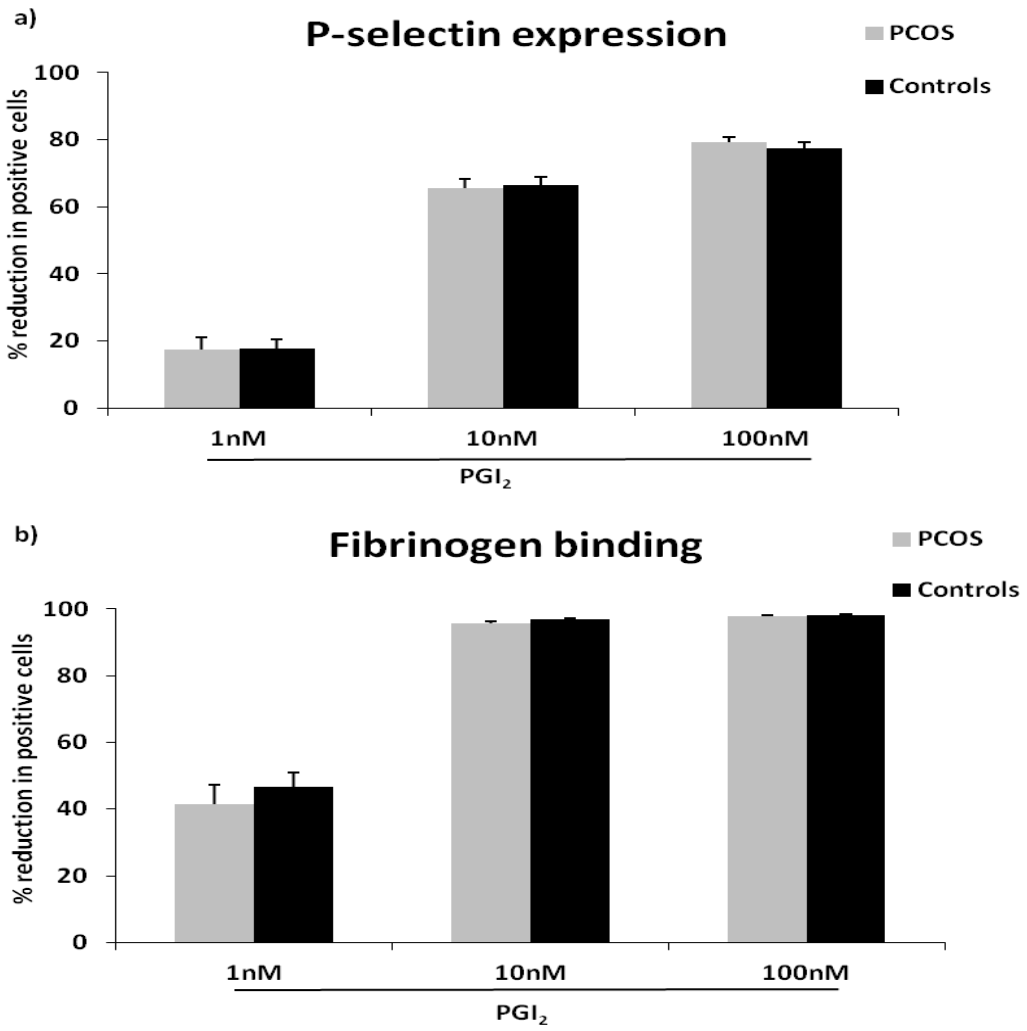


Figure 2.12 Platelets response to the inhibitory effects of PGI_2 in the PCOS and control groups. Fig 2.12a Percentage reduction in P-selectin expression when samples were incubated with PGI_2, at specified doses, for 90 seconds before stimulation with ADP 10µM and fixing at 20 minutes. **Fig 2.12b** Percentage reduction in fibrinogen binding when samples were incubated with PGI_2, at specified concentrations, for 90 seconds before stimulation with at ADP 1µM and fixing at 5 minutes. Data are presented as mean ±SEM. Differences between the two groups were not significant P >0.05.

2.4.5 Endothelial function.

Endothelial function data were available on 19 PCOS and 17 controls, as a consequence of four participants being lost to follow up after the initial cIMT. Endo-PAT was not significantly different between the two groups, RHI was 1.9 ±0.5 vs. 1.8 ±0.5 (P=0.61) for PCOS and controls, respectively. Similarly, there was no significant

difference in serum markers of endothelial function (sE-selectin, sP-selectin, sICAM, and sVCAM) between the two groups, data summarised in Table 2.5.

	PCOS (n=19)	Controls (n=17)	P
RHI	1.9 ±0.5	1.8 ±0.5	0.61
sE-selectin (ng/ml)	19 ±10	20 ±12	0.95
sP-selectin (ng/ml)	129 ±4	126 ±26	0.83
sICAM (ng/ml)	303 ±53	302 ±37	0.96
sVCAM (ng/ml)	3384 ±379	3449 ±404	0.62
Urinary isoprostanes (ng/ml)	16.0 ±4.4	11.8 ±7.1	0.04

Table 2.5 Clinical and biochemical markers for endothelial function, and oxidative stress for the PCOS and control groups. RHI, reactive hyperemic index; sICAM, soluble intercellular adhesion molecule-1; sVCAM, soluble vascular cell adhesion molecule-1.

2.4.6 Clot function and fibrinolysis.

Clotting data were available on 19 PCOS and 17 controls. On turbidimetric analysis there was no significant difference in clot MA 0.39±0.1 vs. 0.39±0.1 arbitrary units (AU) (P=0.75), LT 768±198 vs. 740±119 sec (P=0.61) or LA 599±289 vs. 522±188 AU (P=0.36), for the PCOS and control groups, respectively.

2.4.7 Risk of diabetes and metabolic syndrome.

Women with PCOS were at increased risk of diabetes. Seven women in the PCOS group had impaired glucose regulation (IGR) on OGTT (two IFG, and five IGT), compared to one IGT in controls; 33.3% vs. 5.3% (P=0.02). There was no difference in the presence of the metabolic syndrome; eight vs. six; 42.1% vs. 35.3% (P=0.67) for the PCOS and control groups, respectively.

2.5 Discussion.

In this study we have shown that cIMT, platelet, endothelial and clotting functions are not significantly different in obese young women with PCOS compared to weight matched controls. These findings are important as although PCOS has been

linked to obesity, it is not yet clear whether PCOS is associated with increased CV risk above that due to obesity, body fat distribution and associated CV risk markers.

We found no significant difference in average mean or maximum cIMT between PCOS and controls. Our results are in agreement with data by Talbot et al. (150) who studied the largest cohort of patients (125 women with PCOS vs. 142 age matched controls) and found no difference in cIMT between the two groups; only middle aged women with PCOS (≥45years) had higher cIMT compared to controls (150). However, a link between PCOS and increased cIMT has been suggested in other studies (126, 319). Orio et al.(126) found PCOS was associated with increased cIMT when they examined 30 normal weight young women with PCOS and age and weight matched controls. Luque-Ramírez et al. (319) studied 40 young women with PCOS and compared them to 20 matched controls. The authors found PCOS to be associated with increased cIMT independent of obesity and directly related to hyperandrogensim (319). Our data suggest that PCOS does not affect cIMT independently of BMI in obese premenopausal women. In contrast to other studies we only included obese young women with PCOS and matched controls. The PCOS and control groups, in our study, were not only matched for age and BMI, but also for abdominal obesity (waist circumference and WHR), blood pressure, lipids profile, and smoking history, markers which are known to affect cIMT (131). As atherosclerosis is a slow process, it might be that the participants' young age and good health have masked any significant difference between the two groups. It will be of interest to follow these women up to see if the relationship between PCOS and cIMT changes as they become older.

This is the first study to control for confounding obesity when assessing platelet function in PCOS. In this study we assessed both activatory and inhibitory pathways that regulate platelet function and found no difference between PCOS and controls. Dereli et al. (191) compared lean women with PCOS to matched women with NC-CAH and normal controls. Platelet aggregation was found to be higher in the PCOS group and was related to IR as no difference was found between the NC-CAH and control groups. More recently, Rajendran et al. (125) found platelet aggregation in response to ADP to be higher and platelets' responsiveness to NO to be lower in a

group of lean (n=12) and obese (n=12) women with PCOS compared to healthy controls (n=12). The authors related impaired platelet function in PCOS to hyperandrogenism as no difference in platelet aggregation was found between the lean and obese PCOS groups. In comparison to the previous two studies we controlled for obesity when examining platelet function in obese women with PCOS. While all ex-vivo studies of platelet function are prone to artifactual platelet activation, whole blood flow cytometry has the advantage of directly analysing individual platelets with high degree of sensitivity and with minimal sample manipulation (284). Inflammation and obesity have been shown, in previous studies, to affect platelet function (193). Our data suggest that PCOS does not significantly affect platelet function independent of obesity. It will be of interest to examine the effect of weight loss on platelet function and to follow these women up to see if this relationship changes when they become postmenopausal.

Similarly, we found no significant difference in the acellular arm of thrombosis, with clot structure parameters showing no difference between obese women with PCOS and controls. Our data support findings by Słopień et al. (320) who suggested that PCOS doesn't significantly affect fibrinolytic activity after examining 19 obese young women with PCOS and 20 matched controls.

Evidence of impaired endothelial function in PCOS is inconsistent. RHI measured by Endo-PAT represents changes in microvascular blood flow which are partly controlled by endothelial derived NO (321). A RHI value of less than 1.67 is thought to reflect endothelial dysfunction (297). Interestingly, in our study, the mean RHI for both groups was higher than that cut off. Our data do not support findings by Lowenstein et al. (322) who compared 31 normal weight young women with PCOS with weight matched controls, using Endo-PAT, and found women with PCOS to have lower RHI: 1.48 ±0.32 vs. 2.00 ±0.51 for the control group (P=0.04) after adjusting for age. However, our data support findings by Ketel et al. (323) who compared 31 obese (18 with PCOS) and 39 lean (22 with PCOS) young women and found central obesity, rather than, PCOS, to be associated with increased arterial stiffness. Similarly, we found no significant difference between the two groups in the levels of endothelial adhesion molecules (sVCAM-1, sICAM-1, sP-selectin, and

sE-selectin), inflammatory marker (hsCRP) or prevalence of the metabolic syndrome. As many of these markers are linked to obesity (268), it is likely that obesity might have masked any independent effect to PCOS on these measures.

Obese women with PCOS, in our study, had higher oxidative stress, IR and were at increased risk of diabetes on OGTT. Our findings are consistent with previously published data (5, 324). IGT has strongly been associated with PCOS and in one study 54% of women with PCOS and IGT developed type 2 diabetes over a follow up period of 6.2 years (325). IR may cause hyperglycaemia and oxidative stress and together may contribute to endothelial dysfunction, dyslipidaemia, inflammation, platelet dysfunction, and atherosclerosis (326). However, despite the increased levels of IR and oxidative stress observed in the PCOS group in our study, all the CV risk markers measured were not different compared to controls. It is possible that the independent effects of PCOS on CV risk in the presence of obesity are either minimal or not yet apparent at a young age; or that there are other markers, yet to be identified, which offer CV protection for women with PCOS and provide a buffer against the increased levels of IR and oxidative stress.

The strengths of this study include that the PCOS and control groups were well matched for age, weight and known CV comorbidities; and we have used well validated methods to extensively assess a wide, though complementary, range of atherothrombotic risk markers. Limitations to our study include that participants were recruited from a tertiary centre clinic and through advertisements in newspapers which may introduce selection bias. However, our study participants were a mixture of clinic and self-referred patients and we believe they represent well the population of interest. Another limitation is that we have only used one clinical method to assess endothelial function. Endo-Pat is an established method to assess endothelial function, however, it is an expensive test and its signal could be influenced by non-endothelial factors (295). FMD is another established, non-invasive, method to assess endothelial function. However, although reproducible, the test requires special technical expertise and is operator dependent (295). Finally, our study does not completely exclude a small independent effect for PCOS on atherothrombotic risk in obesity as the study was powered to detect a large

effect size. Subsequently a small effect may still exist; although its clinical significance could be arguable.

2.6 Conclusions.

Our data suggest that when women were matched for age and obesity, PCOS did not independently increase atherothrombotic risk markers. This was despite young obese women with PCOS demonstrating more insulin resistance, hyperglycaemia, oxidative stress and hyperandrogenaemia. It is likely that any excess CV risk in young obese women with PCOS can either be attributed to obesity or is not yet apparent at this early stage of the condition.

3. Chapter 3: Glucagon Like Peptide-1 Receptor (GLP-1R); a novel receptor in platelets.

3.1 Abstract.

Few studies have examined the effects of glucagon-like peptide-1 (GLP-1) analogues on atherothrombotic risk. Here we report that blood platelets express the GLP-1 receptor (GLP-1R) which we identified through a combination of biochemical and functional techniques. GLP-1R mRNA expression in human platelets was confirmed by reverse-transcriptase polymerase chain reaction (RT-PCR). Immunoblotting demonstrated the presence of a 56kDa protein in platelets and megakaryocytes, which was indistinguishable in terms of size from GLP-1R in endothelial cells. Liraglutide, a GLP-1R agonist, inhibited collagen- and thrombin-induced platelet aggregation, which was associated with increased protein kinase A (PKA) activity. Importantly, the inhibition produced by liraglutide was reversed by the GLP-1R-specific antagonist exendin 9-39. The results suggest that platelets express the GLP-1R and that liraglutide inhibits platelet activation at least partially through the activation of GLP-1R. Treatment with GLP-1R agonist, liraglutide, may have a cardioprotective effect in people with type 2 diabetes and/or obesity.

3.2 Introduction.

Glucagon-like peptide-1 (GLP-1) is a hormone secreted from the L-cells of the small intestine which stimulates glucose-dependent insulin response from the β-cells of the pancreas (209). Following its release into the circulation, GLP-1 is rapidly degraded to its inactive form by the enzyme dipeptidyl peptidase IV (DPP-IV) (205). This rapid inactivation of GLP-1 contributes to a half-life of less than 2 minutes and limits its clinical use (215, 217). Liraglutide is a GLP-1 analogue that was obtained by the substitution of Lys 34 to Arg, and by addition of a C16 fatty acid at position 26 using a Ɣ-glutamic acid spacer (253). Liraglutide has a half-life of 13h that is most likely to be mediated via a lower susceptibility to metabolism by DPP-IV and by binding to albumin (253). Liraglutide is licensed for the management of patients with type 2 diabetes (T2DM) and has been found to improve outcomes after experimental myocardial infarction in mice (265).

GLP-1 receptor (GLP-1R) is expressed throughout the mouse cardiovascular system including cardiomyocytes, endothelial and vascular smooth muscle cells of the normal adult mouse heart (266). GLP-1R has also been found in human umbilical endothelial cells (HUVECs) (327). Its presence and function in platelets are unknown. In this chapter, the expression and function of GLP-1R in human platelets will be investigated.

3.3 Materials.

A detailed list of buffers used and their components is provided in Appendix I; antibodies, agonists and inhibitors used are provided in Appendix II.

3.4 Study participants and methods.

The study was approved by local Ethics Committee and all study participants signed a confirmed consent form before participation. Study participants were healthy and not taking any medication.

3.4.1 Isolation of human platelets.

Principle: Platelets are isolated from whole blood by centrifugation. Citric acid or prostaglandin E_1 (PGE_1) is added to platelet-rich plasma (PRP) to prevent platelet activation during centrifugation.

Procedure: Blood was collected using a 21 gauge butterfly, without stasis. The first 2ml of blood were discarded to avoid any artifactual platelet activation by thrombin generated during venipuncture. Blood was collected into a syringe prefilled with acid citrate dextrose (ACD) buffer (Appendix I) as anticoagulant (4:1, vol:vol). PRP was obtained by centrifugation (Universal 320, Hettich) of whole blood at 200g at 20°C for 20 minutes. PRP was either treated with citric acid 0.3mM, at a ratio of 20µl:1ml PRP to lower PH to 6.4 and prevent platelet activation when handling (PH method) (328), or treated with PGE_1 50ng/ml (PGE_1 method) (329), and then centrifuged at 800g at 20°C for 12 minutes. The platelet poor plasma (PPP) was discarded and the platelet pellet was resuspended in 1ml of modified Tyrodes buffer (Appendix I).

3.4.2 Counting of isolated platelets.

Principle: The concentration of platelets in the suspension is determined to standardise experimental conditions.

Procedure: 5µl of isolated platelets were diluted in 500µl (1:100) of ammonium oxalate (1% w/v). Ammonium oxalate lyses erythrocytes while platelets remain intact. 10µl of the preparation are added to each side of the haemocytometer (Neubauer cell counting chamber) and left for 10min to rest. Platelets are counted under the microscope, X 40 magnification, in five squares on each side of the haemocytometer, each square volume is 0.004µl. Platelet concentration (platelet/ml) is determined by multiplying the number of platelets counted by 25000, to adjust for volume, and 100, to adjust for 1:100 dilution. The isolated platelet suspension is then diluted in modified Tyrodes buffer to a final concentration of 2.5×10^8 platelets/mL, unless stated otherwise.

3.4.3 Platelet aggregation.

Principle: When platelets are activated they form aggregates, the size of which is dependent on the degree of activation. Aggregation is assessed by measuring light transmission through a stirred platelet suspension (isolated platelets or PRP). Platelet activation is induced by the addition of an agonist. Activated platelets may first change shape, leading to a decrease in light transmission, before the initiation of aggregation, leading to an increase in light transmission. Light transmission through the platelet suspension is compared to a blank (either modified Tyrodes buffer or PPP), representing maximum light transmission (Figure 3.1). Platelet aggregation was first described in 1962 (330) and is still widely used for assessing platelet function.

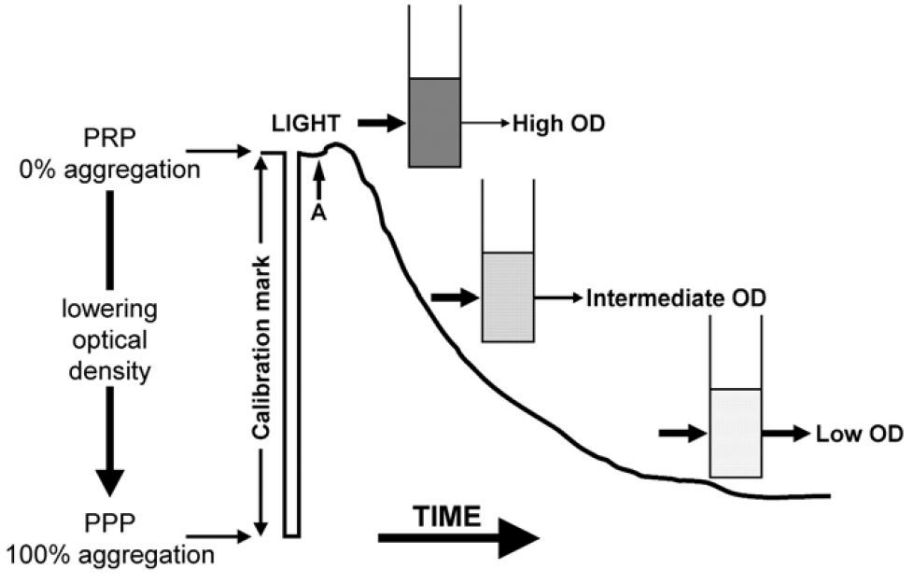


Figure 3.1 Diagram of a platelet aggregation trace. The unstimulated suspension of PRP has a relatively high optical density, which represents 0% aggregation. Following addition of the agonist (A) the platelets aggregate, allowing more light to pass through the suspension of platelets and resulting in a reduction in the optical density. PPP suspension provides the measured optical density equivalent to 100% aggregation as indicated by the calibration mark. Obtained from Platelets and Megakaryocytes (331).

Procedure: Aggregation was monitored using a Chrono-log dual-channel light aggregometer. Isolated platelets (3×10^8 platelets/mL) or PRP were incubated with stirring for 1 minute to allow for temperature equilibration and then treated with the required agonist for 3 minutes at 37°C. Percentage aggregation was recorded after 3 minutes from the aggregation traces produced by AggroLink computer software from Chrono-log.

3.4.4 Measurement of protein concentration.

Principle: Platelet lysate protein concentrations were evaluated using the Bio RAD DC protein assay following the manufacturer's instructions. It is a colorimetric assay based on the Bradford protein assay (332), based on proteins reacting with copper in an alkaline solution. The copper bound proteins then reduce Folin's reagent to create a coloured product which can be detected at a wavelength of 750nm. Colour development is mainly due to the amino acids tyrosine and tryptophan, although cystine, cysteine and histidine also react.

Procedure: 100µl of isolated platelets were added to 100µl of lysis buffer (Appendix I). Protein concentration was determined using the Bio-Rad DC Protein Assay kit as per manufacturer's protocol.

3.4.5 Gel electrophoresis and western blotting.

Western blotting (WB) identifies, through specific antibodies, proteins that have been separated from one another according to their size and charge by gel electrophoresis. Any charged ion or group of ions will migrate when placed in an electric field.

Principle:

I. Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE).

Electrophoresis allows the separation of charged macromolecules in an electric field. When applied to a porous matrix such as a gel it can be used to separate molecules based on their size and charges. Sodium dodecyl sulphate - Polyacrylamide Gel Electrophoresis (PAGE), uses a combination of SDS and the polyacrylamide gels to separate proteins according to their molecular masses by

electrophoretic migration. SDS is an anionic detergent that binds to proteins in a constant weight ratio (1.4g of SDS per gram of polypeptide) and denatures proteins leaving them with similar, rod-shaped tertiary structure. The intrinsic charges of the polypeptide are insignificant compared to the negative charges provided by the bound detergent, so that the SDS-polypeptide complexes have essentially identical charge densities and migrate in polyacrylamide gels of the correct porosity according to polypeptide size.

The polyacrylamide gel results from the polymerization of acrylamide monomer into long chains and the cross linking of these by bifunctional compounds such as N, N'-methylene bisacrylamide reacting with free functional groups at chain termini. Polymerization of acrylamide is initiated by the addition of ammonium persulphate and N, N, N', N'-tetramethylenediamine (TEMED). TEMED catalyses the formation of free radicals from persulphate and these in turn initiate polymerization. Polyacrylamide gels, in addition to preventing heat convection and minimizing diffusion, they also help in separating protein molecules through their pore sizes (sieving effect). The effective pore size of polyacrylamide gels is greatly influenced by the total acrylamide concentration in the polymerization mixture, effective pore size decreases as acrylamide concentration increases.

II. Immunoblotting.

To identify specific proteins separated from the mixture, immunoblotting is routinely used. This involves the transfer of the separated proteins from the gel into a thin support matrix under an electrical field (333). Once transferred, the membranes are probed with specific primary antibodies against target proteins. This is followed by incubation with an enzyme-conjugated secondary antibody, usually horseradish peroxidase (HRP). Detection of antigen-bearing proteins is facilitated by enhanced chemiluminescence (ECL) through which a signal can be produced as a result of an interaction between hydrogen peroxide and luminol in the presence of horseradish peroxidase. The outcome of this interaction is an excited product, which decays to a lower energy state and simultaneously luminesces at 425nm that can be captured and visualised on X-ray films.

Procedure:

I. Sample preparation.

Samples preparation was adapted from the method described by Laemmli (334). After the isolation of isolated platelets, platelet samples were incubated at 37°C with stirring. Agonists and/or inhibitors were added as specified at each experiment. The reaction is terminated by the addition of Laemmli buffer (Appendix I), to isolated platelet samples (1:1 vol:vol). Samples were then kept at -20°C untill later analysis.

II. Protein separation by electrophoresis.

On the day of analysis the resolving gel, polyacrylamide 10%-18% gradient, was prepared (Appendix I). The resolving gel was poured with aid of a gradient mixer and a peristaltic pump and left to set at room temperature for approximately 1h. Once polymerised, a 3% stacking gel (Appendix I) was poured on top of the resolving gel and a well-forming comb was inserted and left for approximately 20 minutes. Once polymerised, the well-forming comb was removed and wells were washed with running buffer (Appendix I). Platelets samples were prepared by heating to 100°C for three minutes. An equal amount of protein (20µg) was loaded to each well. A 10µl of biotinylated protein standard, protein ladder, was loaded to aid in estimation of the molecular weight of separated proteins. Gels were subjected to 120 volts for 2.5h.

III. Immunoblotting.

The polyvinyl-difluoride (PVDF) membrane was immersed in methanol for 3 minutes, to activate the membrane, followed by a 10min wash with dH_2O and finally stored in transfer buffer (Appendix I) till protein transfer. Following protein separation, the resolving gel was inserted into the transfer cassette together with the PVDF membrane, wet blotting paper, and sponges. The transfer cassette was then inserted into the transfer tank, which was filled with transfer buffer. The cassette in the tank is positioned so that negatively charged proteins on the gel migrate towards the anode, so they could be captured by the membrane (Figure

3.2). The transfer tank was placed in an ice box to prevent overheating during transfer. A constant voltage (100v) was applied for 2.5h.

The membrane was then immersed in 10% (w/v) BSA (Appendix I) for 30minutes, to block unoccupied protein binding sites on the membrane. The membrane was then incubated with the primary antibody (1:1000 in 2% BSA) overnight at 4°C with gentle agitation. Subsequently, the membrane was washed twice with Tris base saline-tween (TBS-T) (Appendix I) for 10 minutes before incubation with anti-rabbit or anti-mouse secondary antibody (1:10000 in TBS-T) for 1h at room temperature. Secondary antibody solutions also contained HRP-conjugated anti-biotin antibody (1:1000) for the detection of the biotinylated protein standard. WB membranes were then washed 4 to 6 times, 15 minutes each, in TBS-T before immersing them in ECL1 and ECL2 solutions (Appendix I) for 90sec with gentle agitation protected from light. Membranes were then transferred to an exposure cassette and signal was captured on an X-ray film which and visualised using developer and fixer solutions (Appendix I).

IV. Membrane stripping.

To assess for equal protein loading, the membrane bounds antibodies were stripped by incubating membranes with RestoreTM WB stripping buffer (Thermo Scientific, UK) with agitation for 20 minutes at room temperature. This was followed by two washing steps, with TBS-T and re-blocking with 10% (w/v) BSA for 30 minutes. Membranes were then incubated with anti-β-tubulin antibody (1:1000) overnight at 4^{o}C before membrane development, as described before, on the next day.

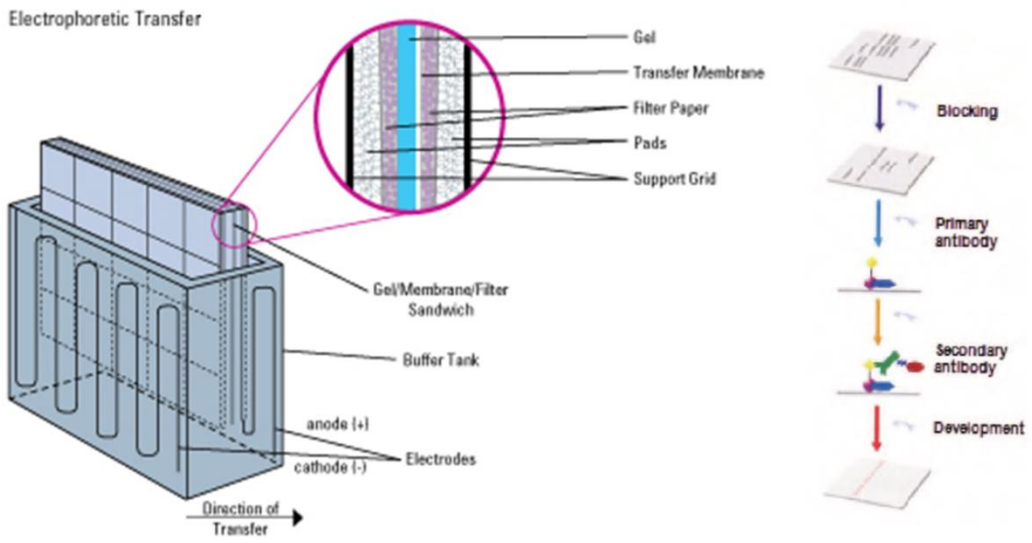


Figure 3.2 A schematic diagram of western blotting method. After protein separation and transfer through the SDS-PAGE method, the membrane is blocked and probed with primary and then secondary antibodies before development on a photographic film (335, 336).

3.4.6 Flow cytometry.

Principle: Flow cytometry measures the properties of individual particles. The sample is aspirated and ordered into a stream of single particles or cells. Each particle passes through beams of laser at the interrogation point. The light scatter is dependent on the particle size and granularity.

When the particle is labelled with a fluorochrome the particle will fluoresce upon excitation by the laser. This fluorescence is converted to a digital signal that can be quantified (Figure 3.3). Data are presented as percentage of positive cells or median fluorescence intensity (MFI). While all *ex-vivo* studies of platelet function are prone to artifactual platelet activation, whole blood flow cytometry has the advantage of directly analysing individual platelets with high degree of sensitivity, in their native environment, and with minimal sample manipulation (284).

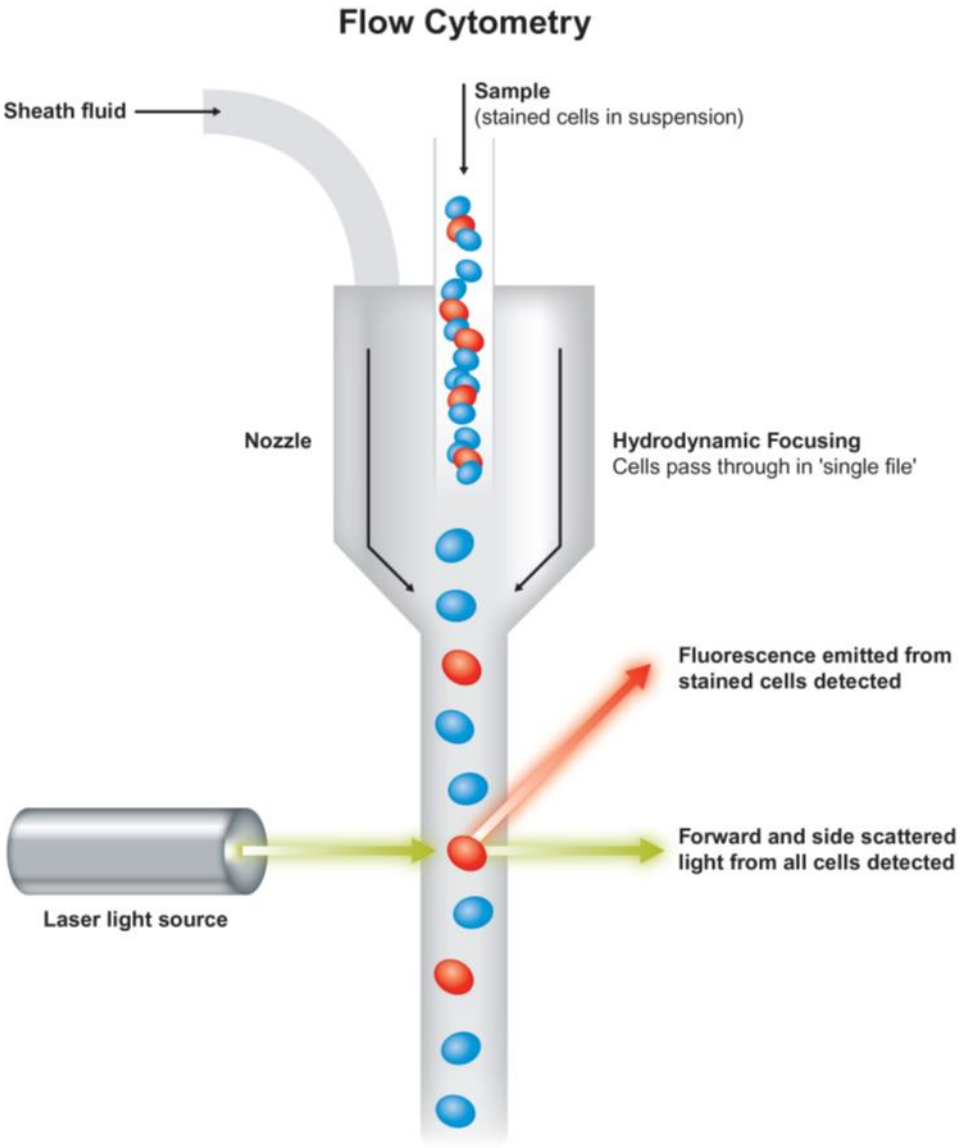


Figure 3.3 A diagram demonstrating the principles of flow cytometry. When the stained cell sample in suspension buffer is run through the cytometer, it is hydrodynamically focused, using sheath fluid, through a very small nozzle. The tiny 'stream' of fluid takes the cells past the laser light one cell at a time. There are a number of detectors to detect the light scattered from the cells/particles as they go through the beam. There is one in front of the light beam (Forward Scatter or FS) and several to the side (Side Scatter or SS). Fluorescent detectors are used for the detection of fluorescence emitted from positively stained cells/particles. The

detectors convert the light and fluorescence signals detected into electronic signals that can be visualised on the computer screen (337).

Procedure: Platelet function was analysed in whole blood by flow cytometry according to method of Goodall et al. (292). Whole blood was collected, as described earlier, into a 3.8% sodium citrate (Appendix I). Within 5min of blood collection, 5µL of citrated blood was diluted in 50µL of modified Tyrodes buffer (Appendix I) and mixed with 5µL of fluorescein isothiocyanate-conjugated (FITC) or phycoerythrin-conjugated (PE) appropriate antibody (Appendix II). Samples were fixed with 500µl of 0.2% paraformaldehyde (Appendix I) at 20 minutes, unless stated otherwise, and analysed within 1 hour by flow cytometry. The platelet population was identified by forward (FSC) and side (SSC) scatter and confirmed by the expression of platelet-specific surface marker CD42b. Fibrinogen binding and P-selectin expression were calculated from 10,000 platelet events. In some experiments whole blood was incubated with agonists and/or inhibitors, as specified in each experiment, before fixation with 0.2% paraformaldehyde and analysis, as described earlier. While examining fibrinogen binding and P-selectin expression on platelet surface in unstimulated samples provide an assessment of platelet function *in vivo*, inclusion of an exogenous agonist +/- inhibitor in the assay enables analysis of the reactivity of circulating platelets *in vitro* (284).

When examining isolated platelets, a similar procedure was performed with the exception of adding 5µl of isolated platelets (1×10^8 platelets/mL), prepared as described before, instead of whole blood, to modified Tyrode's buffer.

To examine white blood cells (WBC) in whole blood, the WBC population was identified by FSC and SSC and confirmed by the expression of the WBC-specific surface marker CD45. 50µl of whole blood were diluted in 50µl of modified Tyrode's buffer and mixed with 5µl of the appropriate antibody before fixing samples with 1mL of lysis buffer at 20 minutes. Samples were further diluted by the addition of 500µl of modified Tyrode's buffer 10 minutes before analysing by flow cytometry.

3.4.7 Phospho-flow.

Principle: Phospho-flow enables the detection of intra-cellular proteins using flow cytometry. Cells are initially fixed in order to minimize leakage of proteins out of the cell. Cells are then permeabilised, allowing conjugated antibodies access to proteins within the cell, and incubated with the appropriate antibody. Following a final wash, the cells were analysed by flow cytometry.

Procedure: The method was adapted from a protocol described by Krutzik et al. (338). Isolated platelets (200µl; 3×10^8 platelets/mL), were fixed with 200µl of 4% formaldehyde (final concentration 2%) and left undisturbed at room temperature for 10 minutes before centrifugation at 1000g for 10 minutes at 4^0C. The platelet pellet was resuspended in 200µl of perm buffer (0.2% triton X-100) and left on ice for 10min before centrifugation at 1000g for 10 minutes. The pellet was resuspended in 100µl of cold phosphate buffered saline (PBS) and kept on ice. 10µl of appropriate FITC or PE-labelled antibody was added to each tube. The samples were kept in the dark on ice for 30 minutes. The samples were then centrifuged at 1000g for 10 minutes and platelet pellet was resuspended in 200µl of cold PBS. Samples were then analysed using flow cytometry as described earlier.

3.4.8 Culture of Human Umbilical Vein Endothelial Cells (HUVECs).

Principle: Cells are allowed to grow, in vitro, under controlled conditions. With the exception of some cells derived from tumours (immortalized cell line), most primary cell cultures have a limited lifespan and after a few divisions may lose their functional and genetic characteristics (339).

Procedure:

I. Initiating and maintaining cultures from cryopreserved cells.

Pooled Human Umbilical Vein Endothelial Cells (HUVECs) were obtained from Life Technologies Corporation, California, USA. 24h prior to cell culture, a T-75 flask was prepared by adding 4ml of GIBCO Attachment Factor (AF), a sterile solution (1x) containing gelatin, and the flask was rocked to ensure the AF covers the entire flask bottom surface. The flask was kept in 37^oC, 5% CO_2/95% air, humidified cell culture incubator till next day. On the next day a vial of HUVECs, 1×10^6 (1mL) frozen in

liquid nitrogen, was thawed by placing the vial in the incubator at 37^{o}C for 5 – 10 minutes. Viable cells were counted by taking 20µl from the vial into 20µl of trypan blue solution: trypan blue leaks into cells if their plasma membrane was damaged giving them a blue colour. The number of viable cells/ml was counted using the haemocytometer. The content of the vial (1 mL) was diluted to a concentration of 1.25×10^{4} viable cells/mL by adding the supplemented culture medium, GIBCO DMEM with Low Serum Growth Supplement (LSGS), as per manufacturer's protocol. 1ml of the diluted thawed HUVECs was added to 9ml of supplemented culture medium in a 15ml Falcon tube. The mixture was added to the AF coated T-75 flask, prepared the day before, and kept in 37^{o}C, 5% CO_2/95% air, humidified cell culture incubator. The culture medium was changed at 24 hours and then every 48 hours. After around 5 days the cells reached 80% confluent and were ready for splitting.

II. Preparation of HUVECs lysates.

24 hours prior to cells splitting a 6-well plate was prepared by adding 1ml of AF to each well. To detach the cells from the T-75 flask, 2ml of trypsin/EDTA solution was added and the flask was shaken gently. After 2 minutes the cells were inspected under microscope to ensure they were round in shape and detached. The cells were then dislodged by gentle tapping on each side of the flask. When cells were dislodged, 3mL of trypsin neutralizing solution was added to deactivate trypsin. The cells were then transferred to a 15mL Falcon tube and the volume was made up to 5ml with supplemented culture medium. The cells were centrifuged at 180 X g for 9min and the pellet was resuspended in 6mL of culture media. 1ml of the cell suspension was added to each well of the 6-well plate that was prepared the day before, as described earlier. The 6-well plate was placed in the incubator and medium changed at 24h then every 48h till cells reached 80% confluency. To lyse the cells, 20µl of protease inhibitor cocktail was added to 4mL of lysis buffer (Appendix I) to stop protein degradation. 500µl of the lysis buffer solution was then added to each well of the 6-well plate and HUVECs were detached by scrubbing using a clean rubber policeman. Protein concentration was determined by protein assay as described before. 10% 2-mercaptoethanol and a trace of bromophenol

blue were added to the cells lysate before storing at -20^{o}C till later analysis by western blotting.

3.4.9 Reverse transcriptase polymerase chain reaction (RT-PCR).

Principle: The polymerase chain reaction (PCR) is used to amplify a sequence of deoxyribonucleic acid (DNA) using a pair of oligonucleotide primers each complementary to one end of the DNA target sequence. These are extended towards each other by a thermostable DNA polymerase in a reaction cycle of three steps: denaturation (separation of the duplex DNA), primer annealing (binding), and polymerization (elongation). As platelets do not contain DNA, complementary DNA (cDNA) is generated by reverse transcribing messenger Ribonucleic acid (mRNA) before amplifying by PCR. In contrast to DNA, cDNA only includes exons (expressed genes).

Procedure:

I. RNA extraction from platelets.

The protocol was adapted from the method described by Peyruchaud et al. (340). Platelets were isolated and the pellet was resuspended in 400µl of modified tyrode's buffer (Appendix I). 15µl of ethylene glycol tetraacetic acid (EGTA) 1mM were added to the suspension to avoid platelet aggregation secondary to the high concentration. The platelet suspension was transferred into an Eppendorf and centrifuged at 12,000g at 4^{o}C for 5 minutes. The supernatant was discarded and the pellet was resuspended in 50 – 100µl of RNase-free H_2O and then incubated at 100^{o}C for 5 minutes. The platelet solution was centrifuge at 14,000g at 4^{o}C for 5 minutes. The supernatant, which contains RNA, was aspirated and kept in RNase-free eppendorf at 4^{o}C. Total RNA concentration (ng/µl) was determined by nanospectrophotometer (Implen, Germany). RNA purity was also determined by measuring absorbance 260/280 nm (a value of 1-2 suggests good purity). RNA is then stored at -80^{o}C.

II. cDNA synthesis.

Isolated total RNA was reverse transcribed to cDNA using EZ-First strand cDNA synthesis KIT (Geneflow, Israel) as per manufacturer protocol. These consisted of

mixing 1µg RNA and 2µM Oligo (dT) Primer in a 0.25mL nuclease free eppendorf tube. DEPC-Treated water was used to bring the volume up to 10µl. The mixture was then gently mixed and heated at 70˚C for 10 minutes in a thermocycler. Eppendorf tubes were placed rapidly on ice, 8µl Reaction Mix (2.5X) and 2 µl DTT (100 mM) added to each tube and mixed gently by pipetting up and down. Samples were incubated at 42^{o}C for 60 minutes followed by 15 minutes incubation at 70^{o}C to stop the cDNA synthesis reaction. The cDNA was stored at -20^{0}C till analysis.

III. PCR.

Primers were designed using Primer Blast from the NCBI website and obtained from (Sigma-Aldrich Company Ltd. Dorset, UK). Each primer was 'blasted' to check for its specificity. Details of the primers are given in Table 3.1.

Gene	Primer	sequence	Tm (^{o}C)	Length (bp)
Human β actin	hACTB F	ACAGAGCCTCGCCTTTGC	59.70	209
	hACTB R	GGAATCCTTCTGACCCATGC	59.73	
Human GLP-1R	hGLP-1R F	GGAGACGGTGCAGAAATGGCGA	59.48	71
	hGLP-1R R	AGGTCCGGTTGCAGAACAAGTGG	59.32	

Table 3.1 Solution PCR primers and their sequences. Tm, temperature; bp; base pair; F, forward; R, reverse.

cDNA was amplified using GoTaq® DNA Polymerase kit (Promega Corporation, USA). For each PCR reaction, 1µl of cDNA was added to 1ul of respective forward primer, 1µl of reverse primer, 9.5µl nuclease free water, and 12.5µl of green GoTaq reaction buffer in a sterile, nuclease-free microcentrifuge tube. Samples were amplified in a thermal cycler (Applied Biosystems): one cycle at 95°C for 5min (initial denaturation), 40 cycles at 95°C for 15sec (denaturation), 55°C for 30seconds (annealing) and 1 minute at 72°C (elongation), and one cycle at 72°C for 7 minutes (final extension). PCR products were detected using 2% agarose gel electrophoresis.

3.4.10 Platelet toxicity study.

Aim: The aim of the study was to assess if liraglutide at high doses was toxic to platelets.

Principle: Platelets contain alkaline phosphatase in their lysosomes (341). The enzyme is restricted to these organelles in viable cells. However, following platelet death due to toxicity, platelet cell membranes become compromised and allow leakage of alkaline phosphatase into the surrounding medium. This enzyme can degrade p-nitrophenyl phosphate (neutral colour) to p-nitrophenol (yellow) allowing its quantification through absorbance measurement with a plate reader. The less viable the cells, the deeper the yellow colour.

Procedure: Isolated platelets (2.5×10^8 platelets/mL) were divided into; sample 1 negative control (unstimulated platelets); samples 2, 3 and 4, incubated with increasing doses of the drug to investigate its toxicity; and sample 6 positive control (unstimulated platelets). At 5 minutes and using a 96-well plate, 50µl from each sample was added to 150µl of p-nitrophenyl phosphate (5mM) in citrate buffer (29mM citric acid, 68mM sodium citrate dehydrate, PH: 5.4), apart from sample 6 (positive control) where 50µl of isolated platelets were added to 150µl of p-nitrophenyl phosphate (5mM) in citrate buffer with Triton X-100 (0.1%). Samples were made in triplicates. The 96-well plate was placed on a plate shaker for 1 hour before the addition of 100µl of 2M NaOH to stop the reaction. Light absorbance was then measured at 405nM using a plate reader.

3.4.11 Statistical analysis.

Results are expressed as mean ± standard deviation (SD) and were analysed using dependent t-test. Figures are presented as mean ± standard error of the mean (SEM). A two-tailed P value of <0.05 was considered statistically significant.

3.5 Results.

3.5.1 The expression of GLP-1R in platelets.

In the first instance, we examined whether GLP-1R was expressed in platelets. Using RT-PCR we found GLP-1R gene to be expressed in human platelets and in HUVECs

cells (Figure 3.3a). To exclude contamination with WBC we assayed platelet preparations for the expression of CD45 by flow cytometry and found the solution to be free from WBC (Table 3.2). GLP-1R was also expressed in human platelets when examined by flow cytometry (figure 3.3b) and immunoblotting (Figure 3.3c).

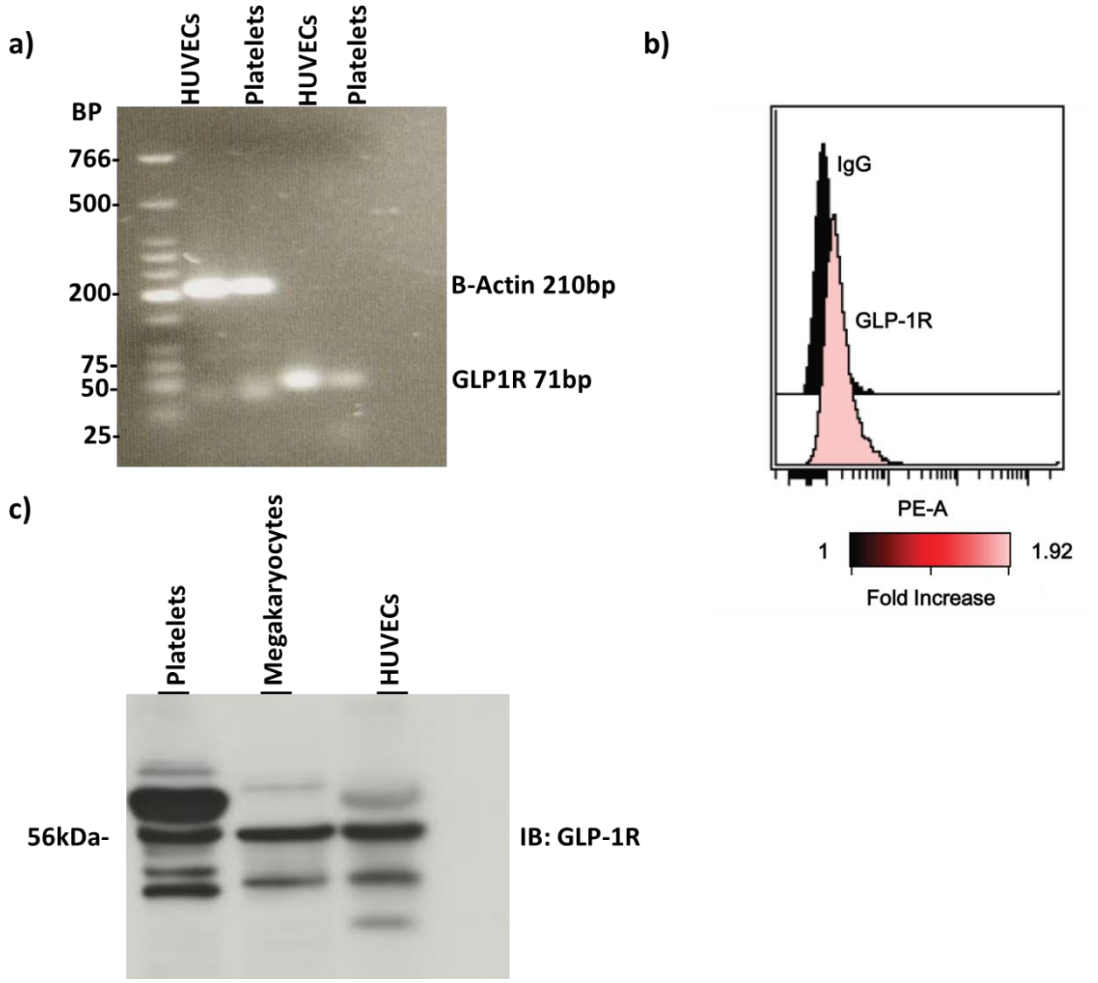


Figure 3.3 GLP-1R expression in platelets. Fig 3.3a Reverse transcriptase-polymerase chain reaction (RT-PCR) amplification products. PCR products were eletrophoresed on a 2% agarose gel, indicating positive expression of β-Actin and glucagon like peptide-1 receptor (GLP-1R) in human umbilical vein endothelial cells (HUVECs) and platelets. Lane 1 shows DNA ladder for bands ranging in size from 25 – 766 base pairs (BP). PCR products were loaded on the gel as follows: lane 2, β-Actin (210bp) in HUVECs; lane 3, β-Actin (210bp) in isolated platelets; lane 4, GLP-1R (72bp) in HUVECs; and lane 5, GLP-1R (72bP) in isolated platelets. **Fig3.3b** Flow

cytometry showing the expression of GLP-1R in isolated platelets. Note the right shift in signal when platelets were incubated with phycoerythrin-conjugated (PE) GLP-1R antibody, compared to the non-specific binding with PE IgG negative control. The colour table represents the change in median fluorescence intensity (MFI). **Fig 3.3c** WB showing GLP-1R (56kDa) expression in platelets, megakaryocytes and HUVECs. Each experiment was repeated three times and the Figure represents an example of the results obtained.

Sample	Antibody	No. of events	% of positive cells	PE-A MFI
Whole blood	IgG neg	44	2.1	100
	CD45	2068	98	353
Isolated platelets	IgG neg	21	2.1	4076
	CD45	6	0.6	3184

Table 3.2 Examining isolated platelet preparations for contamination with white blood cells. Whole blood was used as a positive control and for the identification of the white blood cells (WBC) population. When whole blood sample was incubated with phycoerythrin- (PE) conjugated WBC-specific marker CD45, 98% of the gated cells where positive for CD45 and the median fluorescence intensity (MFI) was 353; in comparison, 2.1% of the cells showed non-specific binding with IgG negative control antibody and MFI was 100. When isolated platelets sample was incubated with CD45: 0.6% of the cells were positive for CD45 (MFI= 3184) compared to 2.1% of the cells positive for IgG negative control (MFI= 4076). As both the percentage of positive cells and MFI recorded in the platelet sample were lower with CD45 antibody compared to IgG negative control, this suggests that any binding for CD45 in the platelet sample was non-specific and that the platelets sample was free from contamination with WBC.

3.5.2 The effects of liraglutide on platelet function.

3.5.2.1 Aggregation in isolated platelets.

Having established the presence of the GLP1R on platelets we began to explore its potential function. Incubating isolated platelets with liraglutide for 1 minute inhibited platelet aggregation in response to collagen and thrombin in a dose-

dependent manner (Figure 3.4). Significant inhibition of both thrombin (0.02U/ml)- and collagen (2µg/mL)-induced aggregation was achieved with liraglutide (50µg/mL), with aggregation being reduced from 62.2 ±6.3% to 32.5 ±20.5% ($p<0.05$) and 60.2 ±8.3% to 23.4 ±17.9% ($P<0.01$), respectively (Figure 3.4).

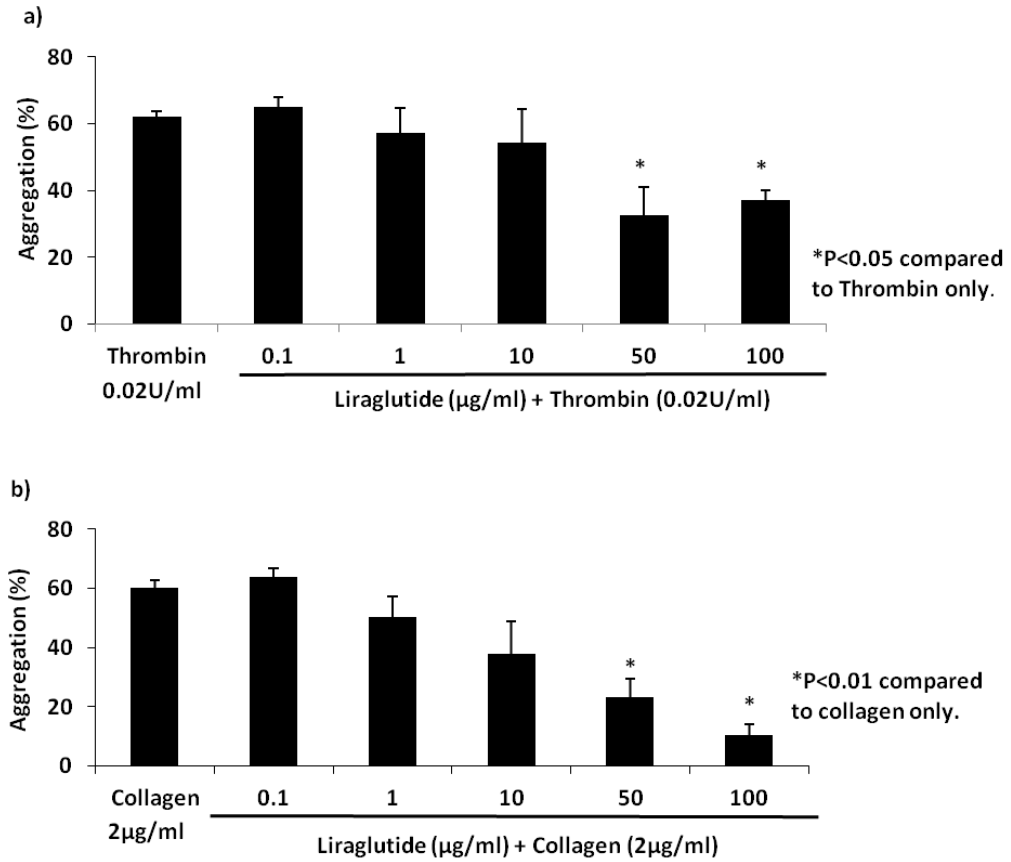


Figure 3.4 Inhibition of isolated platelet aggregation with liraglutide dose-response. Isolated platelets ($2.5x10^8$ platelets/mL) were prepared using PH method. Platelets were incubated with liraglutide, at increasing doses, for 1 minute before adding thrombin (**Fig.3.4a**), or collagen **(Fig.3.4b**). Data presented as mean ±SEM. *P<0.05. N= 6 – 8 independent experiments.

The inhibition of platelet aggregation induced by liraglutide was maximal at 1 – 5 minutes incubation time (Figure 3.5a) and gradually reduced at longer incubation times up to 90 minutes (Figure 3.5b). The inhibition induced by liraglutide, 50µg/mL, was more prominent when platelets were stimulated with lower dose of collagen (1µg/mL; Figure 3.5a) compared to higher dose (5µg/mL; Figure 3.5b). Similarly, stimulating isolated platelets with increasing doses of the agonist reversed the inhibition in platelets aggregation induced by liraglutide (Figure 3.6).

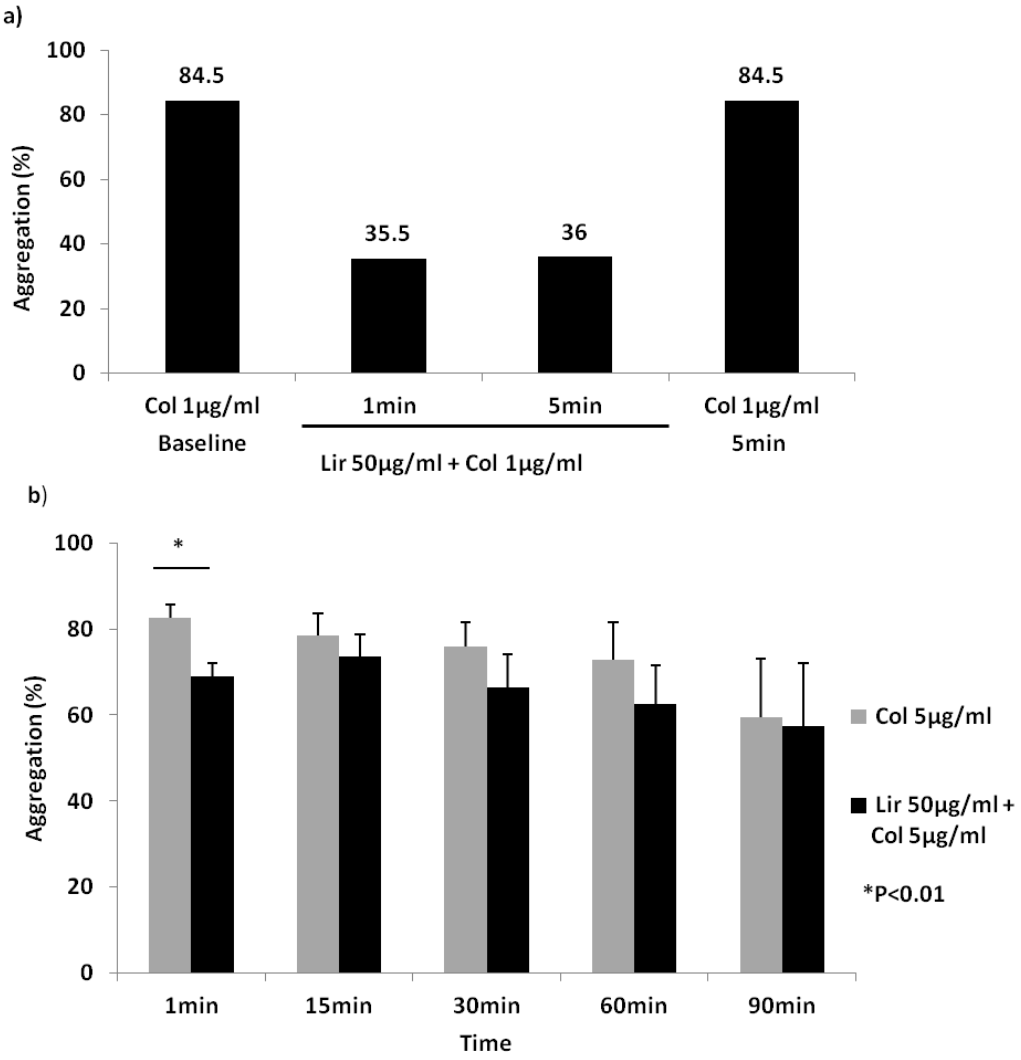


Figure 3.5 Inhibition of platelet aggregation by liraglutide, time-course. Isolated platelets ($2.5x10^8$ platelets/mL) were prepared using PH method. **Fig 3.5a** Samples were incubated with liraglutide 50µg/mL or vehicle for up to 5 minute before stimulation with collagen 1µg/mL. N=2 independent experiments. **Fig 3.5b** Samples were incubated with liraglutide 50µg/ml or vehicle for up to 90 minutes before stimulation with collagen 5µg/mL. N=4 independent experiments. Col, collagen; Lir, liraglutide.

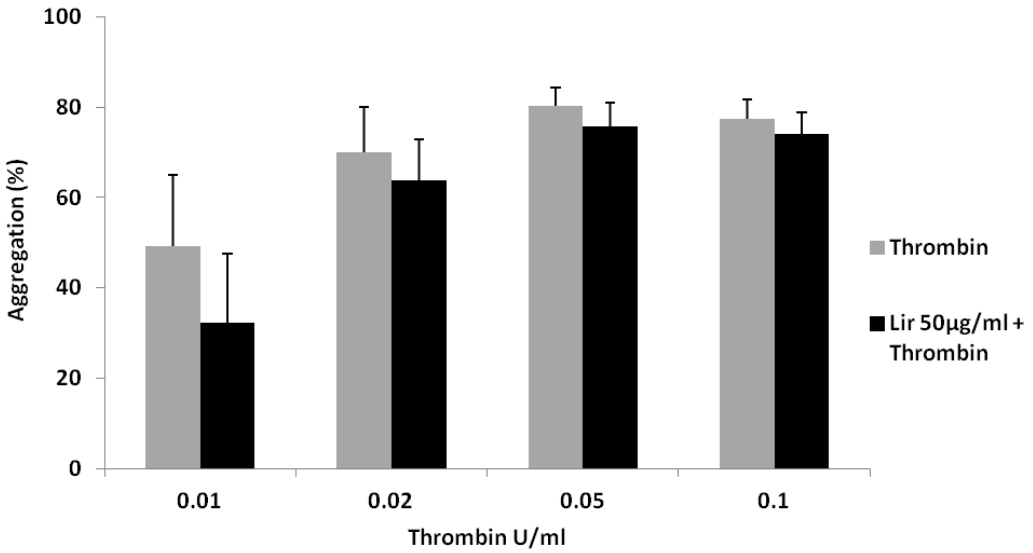


Figure 3.6 Reversal of liraglutide-induced inhibition of platelet aggregation with higher doses of agonist. Isolated platelets ($2.5x10^8$ platelets/mL) were prepared using PGE_2 method. Platelets were incubated with liraglutide 50µg/mL for 1 minute before stimulation with increasing doses of thrombin. N=3 independent experiment. Data presented as mean ±SEM.

As the average concentration of liraglutide in the plasma when given to patients at a dose of 1.8mg od, usual treatment dose, is 0.1 – 0.2µg/mL (255); a toxicity study was performed and showed that liraglutide was not toxic to platelets (Table 3.3).

Sample	Negative control	Liraglutide				Positive control
		0.1µg/ml	1µg/ml	50µg/ml	100µg/ml	
Light absorbance	0.23	0.22	0.22	0.24	0.24	3.96

Table 3.3 Liraglutide toxicity study. Isolated platelets ($2.5x10^8$ platelets/mL), PH method, were incubated with increasing doses of liraglutide or a vehicle for 5minutes. The positive control sample included Triton X-100 (0.1%). Higher light absorbance reflects higher toxicity. N=1.

3.5.2.2 The influence of liraglutide in the presence of plasma.

Incubating PRP with increasing doses of liraglutide (1 – 100µg/mL) for 1 minute did not significantly affect collagen-induced platelet aggregation (Fig 3.7a). Similarly, incubating PRP with liraglutide (50µg/mL) for up to 1 hour did not significantly affect collagen induced platelet aggregation (Fig 3.7b).

To assess if the lack of significant effect for the drug in PRP was due to binding to albumin in the plasma, we re-examined platelet aggregation in isolated platelets with the addition of BSA to modified tyrode's buffer. At a BSA concentration of 1mg/mL, liraglutide 50µg/mL (1 minute incubation) inhibited 4µg/mL collagen-induced platelet aggregation from 56% to 13%. When the concentration of BSA in modified Tyrode's buffer was increased to 40mg/mL, equal to average concentration in human plasma, it was not possible to test the effects of liraglutide as neither collagen nor thrombin induced platelet aggregation even at maximum doses of 10µg/mL and 0.1U/mL, respectively. As increasing the albumin concentration in isolated platelets to plasma levels did not mimic the plasma environment we therefore tested the effects of liraglutide on platelet function in whole blood by flow cytometry.

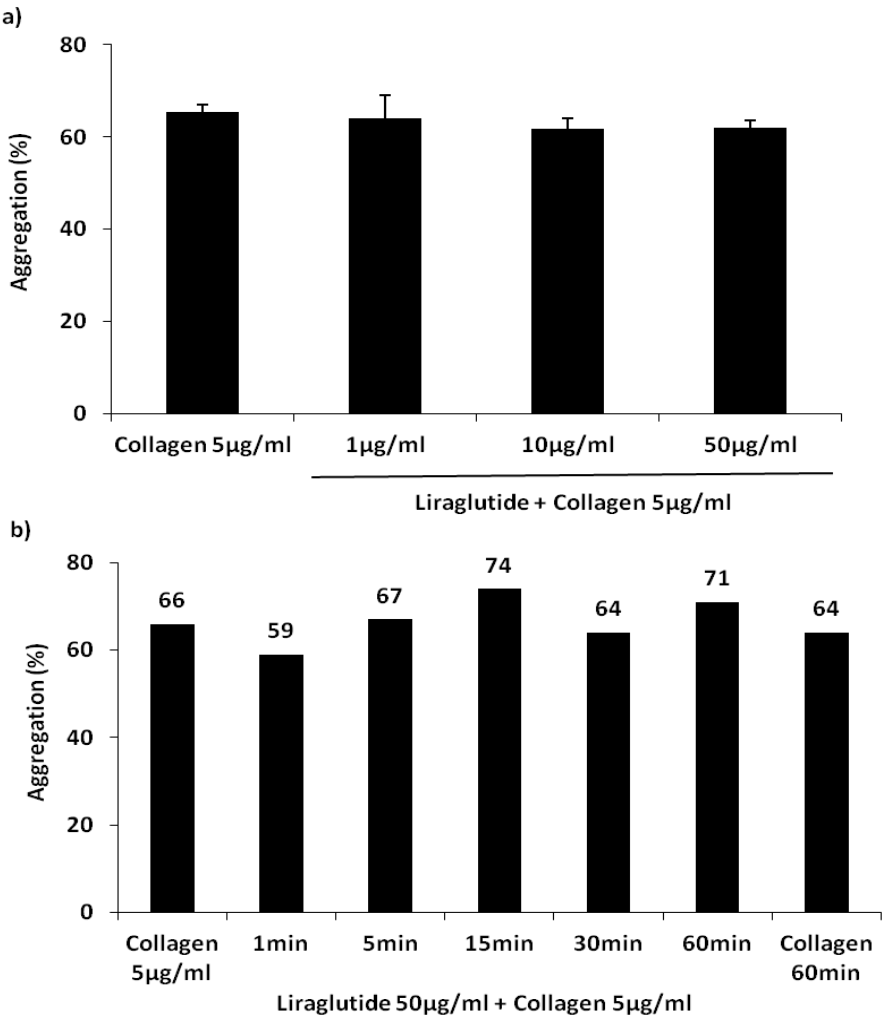


Figure 3.7 Liraglutide effect on platelet aggregation in PRP. Fig 3.7a PRP was incubated with increasing doses of liraglutide, 0.1 – 50µg/mL, for 1 minute before stimulation with collagen 5µg/mL. N=3 independent experiments. Data are presented as mean and error bars represent SEM. **Fig 3.7b** PRP was incubated with liraglutide, 50ug/mL, for up to 60 minutes before the addition of collagen 5µg/mL. N=1.

3.5.2.3 Whole blood by flow cytometry.

Incubating whole blood with liraglutide 50µg/mL for 90sec before stimulation with ADP reduced P-selectin expression on platelet surface (Figure 3.8).

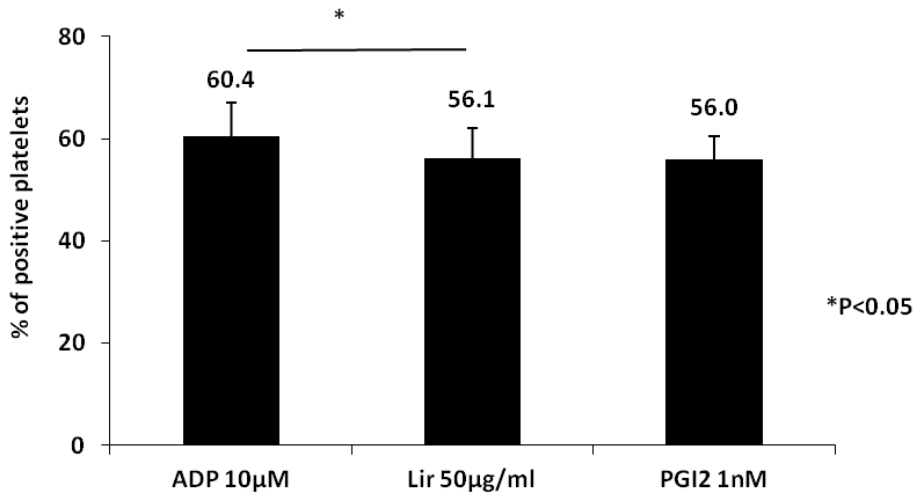


Figure 3.8 Effects of liraglutide on P-selectin expression in whole blood. Whole blood was incubated with liraglutide 50µg/mL or PGI_2 1nM for 90sec, before stimulation with ADP 10µM. Samples were fixed with 0.2% formaldehyde at 20 minutes. Data presented as mean ±SEM. N= 3 independent experiments.

3.5.3 Mechanism of action.

3.5.3.1 Exendin-9-39 partially reverses the effects of liraglutide

Exendin 9-39, is a specific and competitive antagonist of GLP-1Rs (342). It has lower binding affinity to GLP-1Rs than GLP-1 and subsequently 10 – 20 times higher doses of exendin 9-39 are required to inhibit the effect of GLP-1R agonists (265, 343, 344). Incubating isolated platelets with exendin 9-39 (27µM) for 1 minute before the addition of collagen 2µg/mL did not alter collagen induced platelet aggregation. However, exendin 9-39 (27µM) partially reversed the inhibitory effects induced by liraglutide 10µg/mL (2.7µM) in aggregation (Figure 3.9). Here aggregation was reduced from 62 ±7.2% to 35.8 ±15% by liraglutide, but remain elevated at 49.6 ±8.3% in the presence of exendin 9-39 (Figure 3.9).

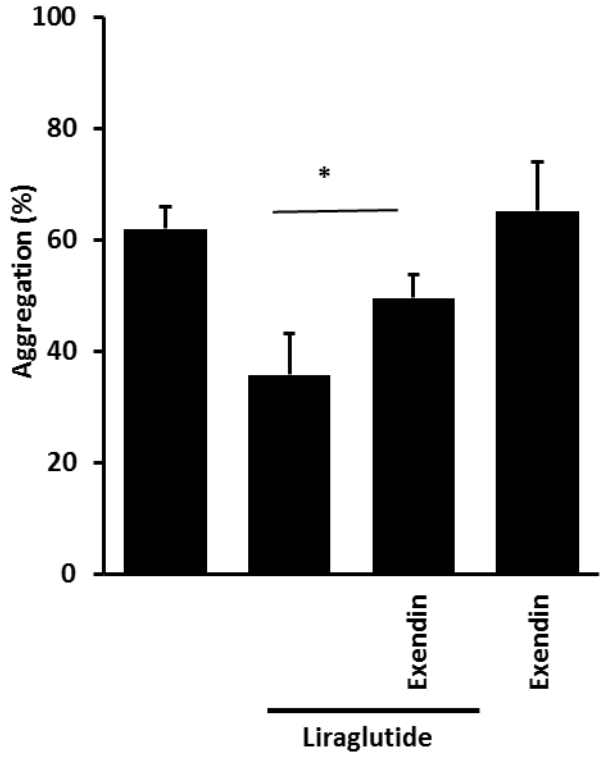


Figure 3.9 Reversal of the effects of liraglutide with exendin 9-39. Isolated platelets, $3x10^8$ platelets/mL, were prepared using PH method. Samples were incubated with Exendin 9-39 (27µM) or a vehicle for 1 minute before the addition of liraglutide 10µg/mL (2.7µM) or a vehicle for 1 minute and stimulation with collagen 2µg/mL. Data presented as mean and error bars represent SEM. N=4 independent experiments.

3.5.3.2 Liraglutide potentiates the inhibitory effects of prostacyclin on platelet aggregation.

Incubating isolated platelets with liraglutide 50µg/mL for 1 minute potentiated the inhibitory effects of PGI_2 on collagen induced platelet aggregation (Figure 3.10a). Platelet aggregation in response to collagen 1µg/ml was reduced by 19% in the presence of PGI_2 1nM, 27% in the presence of liraglutide 50µg/mL, and 64% when PGI_2 and liraglutide were combined (Figure 3.10a). Similarly, liraglutide potentiated the inhibitory effects of PGI_2 on fibrinogen binding on platelet surface, in whole blood, by flow cytometry (Figure 3.10b). Fibrinogen binding in whole blood in response to ADP 1µM was reduced by 8% in the presence of liraglutide 50µg/mL,

20% in the presence of PGI_2 1nM, and 26% when liraglutide and PGI_2 were combined (Figure 3.10b).

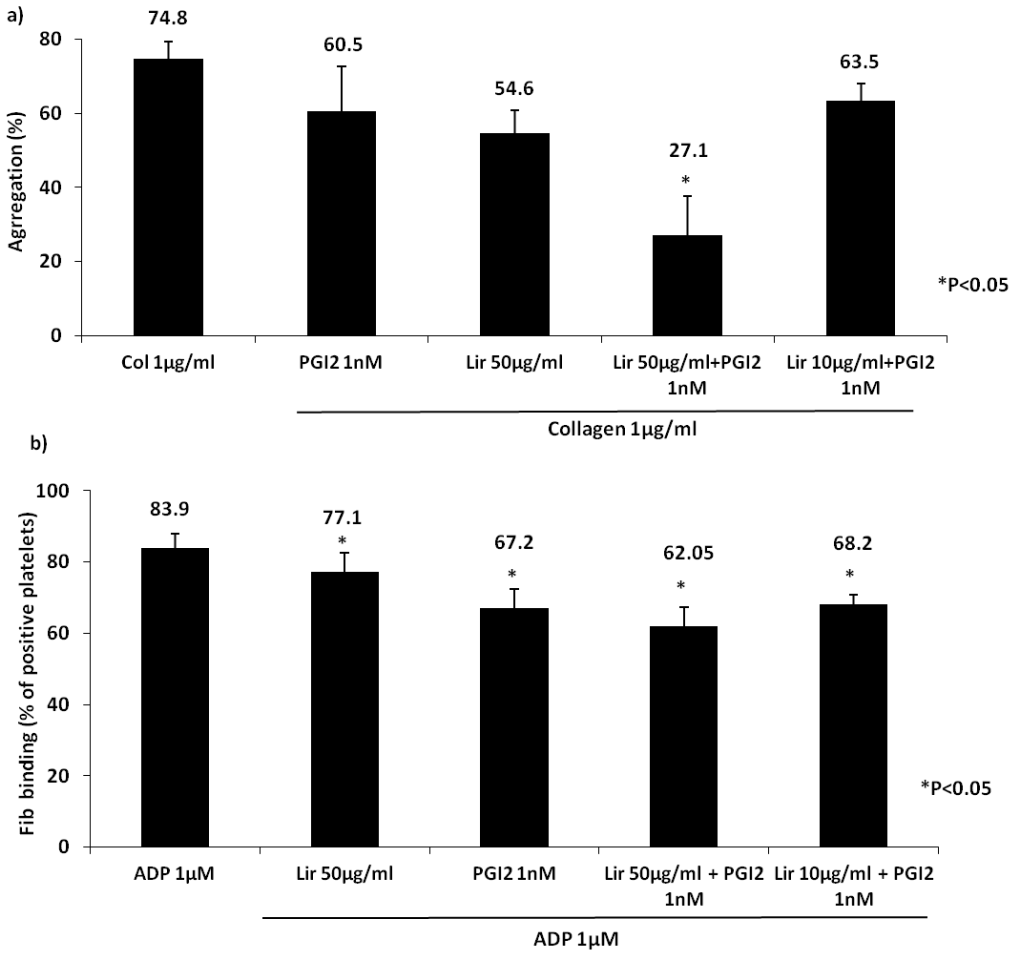


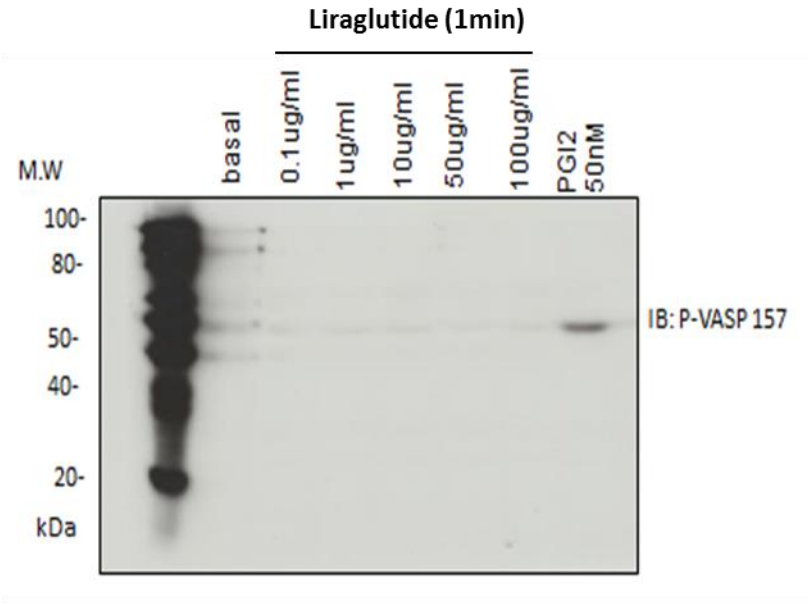
Figure 3.10 Liraglutide potentiates the effects of prostacyclin. Fig 3.10a Effects of liraglutide in aggregation. Isolated platelets ($2.5x10^8$ platelets/mL) were prepared using PH method. Samples were incubated with either a vehicle or liraglutide 50µg/mL for 1 minute before the addition of either PGI_2 1nM or a vehicle for 1 minute and stimulation with collagen 1µg/mL. *P<0.05 compared to either liraglutide 50µg/mL, PGI_2 1nM or collagen 1µg/mL. N= 4 independent experiments. **Fig 3.10b** Effects of liraglutide in whole blood by flow cytometry. Whole blood was incubated with a vehicle, liraglutide (50µg/mL) or liraglutide (10µg/mL) for 1 minute before adding either a vehicle or PGI_2 1nM for 1 minute and stimulation with ADP 1µM. Samples were fixed at 5 minutes by 0.2% formaldehyde. *P<0.05 compared to ADP 1µM. N=6 independent experiments. Data presented as mean and error bars represent SEM.

3.5.3.3 PKA substrates phosphorylation.

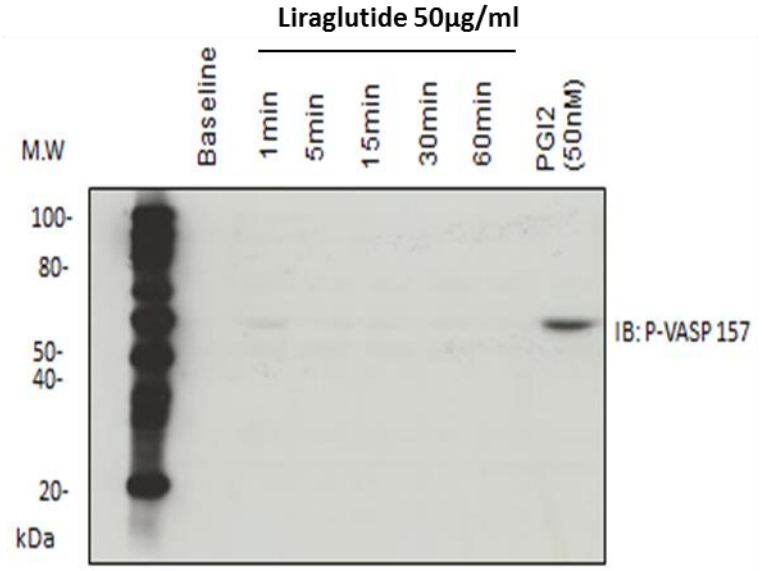
Protein Kinase A (PKA) activation is the main effector of adenylyl cyclase/cAMP downstream signalling in platelets. A common PKA substrate is the vasodilator-stimulated phosphoprotein (VASP) and the main PKA phosphorylation site on VASP is ser^{157} (172). Liraglutide did not increase $VASP^{ser157}$ phosphorylation in immunoblotting or flow cytometry (Figure 3.11a,b,c). Phosphodiesterase 3 (PDE3) is an enzyme that breaks down cAMP and its inhibition results in increased PKA activity represented by enhanced phosphorylation of $VASP^{ser157}$ (176). Subsequently, pre-incubating platelet samples with milrinone, a specific PDE3 inhibitor, may uncover any small changes in VASP phosphorylation. Liraglutide did not increase $VASP^{ser157}$ phosphorylation when platelet samples were pre-incubated with milrinone (Figure 3.11d).

However, when we employed the phosphorylation of PKA substrates as a marker of pathway activity, we found that proteins with apparent molecular weights 10, 45, 80, 100 and 140kDa were mildly phosphorylated under basal conditions (Figure 3.12). Stimulation of GLP-1R with liraglutide (50μg/mL) increased phosphorylation of these basally phosphorylated PKA substrates and induced the phosphorylation of substrates with apparent molecular weights of 50 and 75kDa. These phosphorylated proteins were comparable to those seen when platelets were treated with PGI_2 (50nM), a physiological potent PKA activator (Figure 3.12).

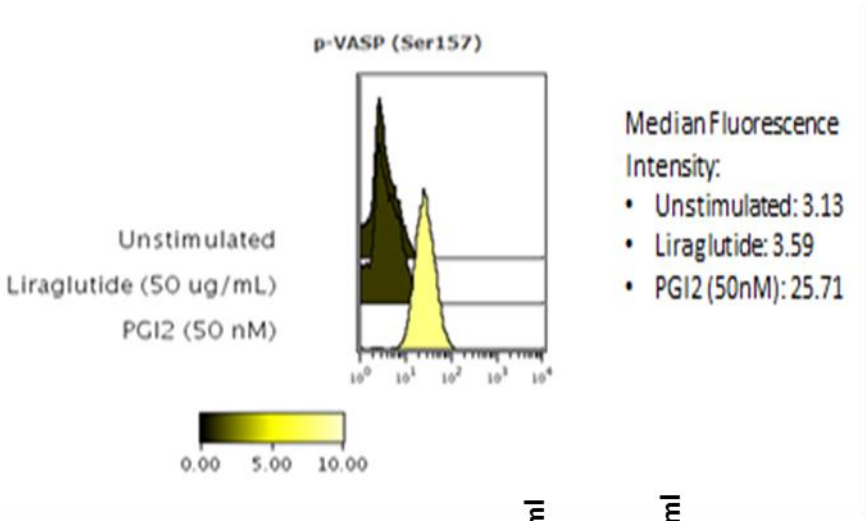
a)


Liraglutide (1min)
basal
0.1ug/ml
1ug/ml
10ug/ml
50ug/ml
100ug/ml
PGI2 50nM
M.W
100-
80-
50-
40-
20-
kDa
IB: P-VASP 157

b)


Liraglutide 50µg/ml
Baseline
1min
5min
15min
30min
60min
PGI2 (50nM)
M.W
100-
80-
50-
40-
20-
kDa
IB: P-VASP 157

c)



d)

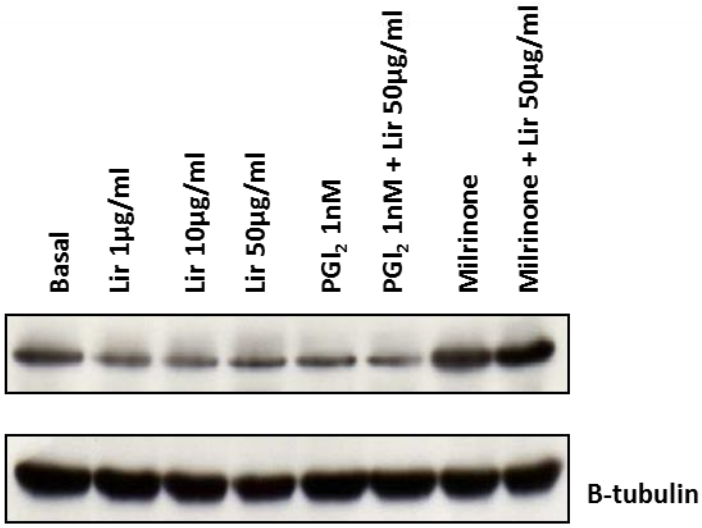


Figure 3.11 Liraglutide does not increase VASP^{ser157} phosphorylation. Isolated platelets were prepared using PH method. **Fig 3.11a** Immunoblotting; samples were incubated with increasing doses of liraglutide 0.1 - 100µg/mL or PGI_2 50nM for 1 minute. **Fig 3.11b** WB; samples were incubated with liraglutide 50µg/mL for up to 1 hour or PGI_2 50nM for 1 minute. **Fig 3.11c** Flow cytometry; isolated platelets ($2.5x10^8$) were incubated with liraglutide 50µg/mL or PGI_2 50nM for 1 minute before fixation. **Fig 3.11d** Immunoblotting; samples were incubated with increasing doses of liraglutide 1 - 50µg/mL or PGI2 1nM, or a combination of liraglutide 50µg/mL and PGI2 1nM for 1 minute. The last two samples were pre-incubated with milrinone (10µM) for 15 minutes before the addition of a vehicle or liraglutide 50µg/ml for 1min. Each experiment was repeated 3 times apart from the flow

cytometry experiment (Fig3.11c) which was repeated twice. The Figure represents an example of the results obtained from these experiments.

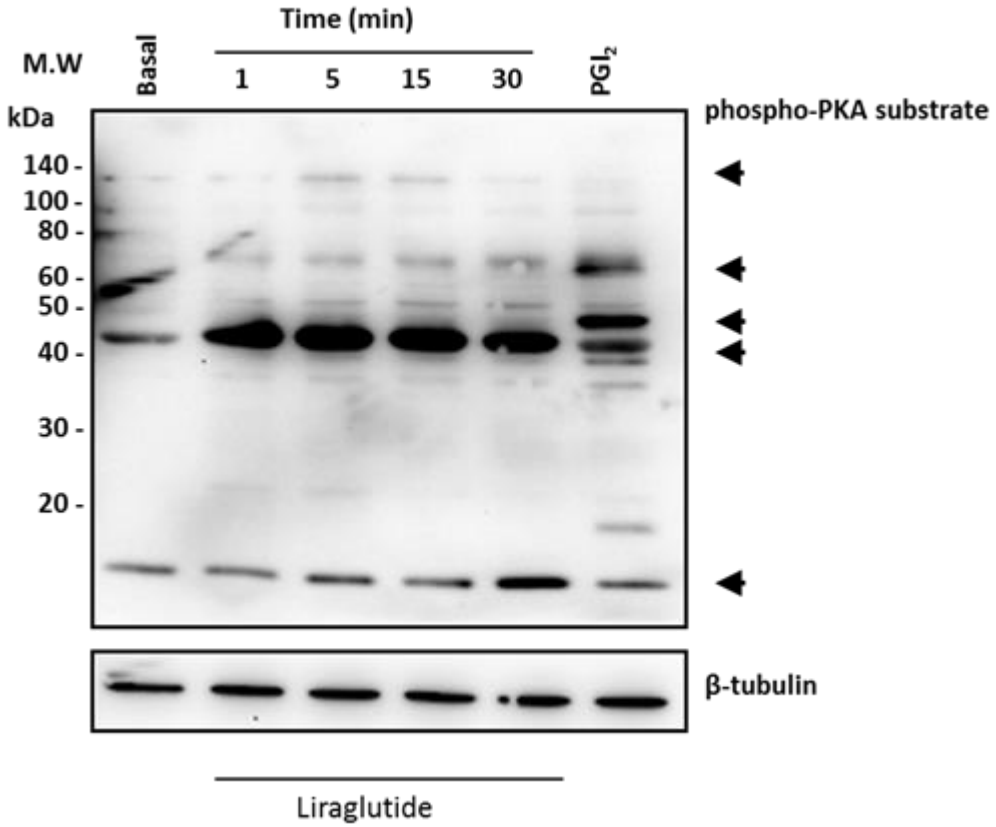


Figure 3.12 Liraglutide increase in PKA substrate phosphorylation. Washed platelets were prepared using pH method. Samples were either incubated with a vehicle for 1 minute (basal), liraglutide (50µg/mL) for up to 30 minutes or PGI_2 (50nM) for 1 minute. Note increase in PKA substrate phosphorylation after incubation with liraglutide (arrows) particularly in proteins with apparent molecular weights 10, 45, 80, 100 and 140kDa. These phosphorylated proteins were comparable to those seen when platelets were treated with PGI_2 (50nM). M.W, molecular weight; kDa, kilodalton; PGI_2, prostacyclin; PKA, protein kinase A. This experiment was repeated 3 times and the Figure represents an example of the results obtained.

3.6 Discussion.

Our data demonstrate the presence of a functional GLP-1R in human platelets and describe some of its cellular effects in these cells. The results also show that liraglutide, a GLP-1 analogue, may have an inhibitory effect on platelet activation. Liraglutide is widely used in the treatment of individuals with type 2 diabetes and is also being investigated as a weight loss medication in simple obesity (114). As people with type 2 diabetes and obesity are at increased risk of cardiovascular (CV) disease (345, 346), our data may have clinical importance since they suggest that liraglutide may have a beneficial effect on platelet function in addition to its role in glycaemic control and weight loss management.

To determine the presence of the receptor on platelets we used a three-pronged strategy. Firstly, RT-PCR was used to confirm the presence of GLP-1R mRNA, which was consistent in size with that from endothelial cells which are known to express the receptor (327). Peripheral blood mononuclear contamination was excluded as platelet samples were negative for CD45, a specific leukocyte marker, by flow cytometry. Immunoblotting of platelet and megakaryocyte lysates with an anti-GLP-1R antibody consistently revealed a platelet protein with an approximate molecular weight of 56kDa, the putative size of GLP-1R. This protein shared identical characteristics to GLP-1R immunoprecipitated from HUVECs when subjected to SDS-PAGE and immunoblotting. This finding was confirmed by flow cytometric analysis in which staining of washed platelets with an anti-GLP-1R monoclonal antibody caused a fluorescence intensity shift compared with isotope control in platelets of three independent donors tested. The reactivity was only found in permeabilised platelets because the antibody used was raised against the intracellular portion of the receptor. However, in other cell types GLP-1R is known to be a surface G-protein-coupled receptor and it is likely that it exists in the same form in platelets. Although, GLP-1R may be expressed throughout the mouse cardiovascular system including cardiomyocytes, vascular smooth muscles and endothelial cells (266, 327), this is the first report of its presence in human platelets.

Having established the presence of the GLP-1R in blood platelets we next examined whether the receptor was functional and what influence its ligation had on platelet function. Incubation of isolated platelets with increasing concentrations of liraglutide (0.1-100μg/mL) for 1 minute led to a concentration-dependent inhibition of collagen- and thrombin-induced aggregation. Our data suggested that liraglutide could induce a modest inhibitory effect on platelet aggregation.

To examine if the effects of liraglutide were mediated through GLP-1R we took advantage of exendin 9-39 a specific and competitive GLP-1R antagonist (342). Exendin 9-39 alone did not induce platelet aggregation or influence aggregation induced by thrombin or collagen. However, pre-incubation of platelets with this small peptide prior to liraglutide (10μg/mL) blocked the inhibitory effects of liraglutide strongly suggesting that liraglutide, at least partly, inhibited platelet aggregation through GLP-1R.

As GLP-1R is a G-protein coupled receptor (Gs) that is known to activate adenylyl cyclase/cAMP pathway and activates PKA, we examined the effects of liraglutide on PKA substrate phosphorylation, the main downstream effectors of adenylyl cyclase/cAMP pathway in platelets (171, 172). Surprisingly, liraglutide did not significantly increase the common PKA substrate $VASP^{ser157}$ phosphorylation. However, there was an increase in other PKA substrate phosphorylation suggesting that liraglutide, consistent with pancreatic β-cells cAMP (264), activates the cAMP signaling pathway which is known to modulate platelet function. In addition, it has been suggested that liraglutide may also work through a GLP-1R-independent pathways including activation of the AMPK signalling pathway (262). In our experiments, the effects of liraglutide on platelet aggregation in isolated platelets remained significant up to 90 minutes incubation time. If the effect of liraglutide noted were merely related to elevation of cAMP, one would expect its effects to fade away much sooner. In addition, exendin 9-39 only partially reversed the inhibitory effects of liraglutide in aggregation suggesting that liraglutide may also by working through a GLP-1R-independent pathway. Interestingly, liraglutide potentiated the inhibitory effect of the natural platelet inhibitor PGI_2 which may represent an additional benefit when given to patients. Our data suggest that the

inhibitory effects of liraglutide on platelet function are, at least partly, mediated through GLP-1R dependent pathway, although an additional GLP-1R independent pathway is also likely.

Interestingly, liraglutide did not significantly affect platelet function in PRP when examined by aggregation. This is likely to be secondary to binding to albumin and subsequent reduction in liraglutide bioavailability and potency. Liraglutide is known to bind albumin reducing degradation by the enzyme DPP-IV in the plasma and increasing its half-life compared to native GLP-1. The fact that the liraglutide remained functional in isolated platelets in the presence of BSA 1mg/mL is reassuring as it suggests that liraglutide may still be functional in the plasma; and the absence of aggregatory effects for collagen and thrombin in the presence of 40mg/mL BSA in platelet preparation is not entirely surprising because in the plasma there are lots of other proteins, lipids and ions to offset the osmotic effect of albumin. Using flow cytometry we showed that liraglutide was still effective in whole blood as evidenced by reduced expression of P-selectin and fibrinogen binding to the platelets' surface when whole blood was incubated with liraglutide 50µg/mL for 2 minutes before stimulation with ADP. The discrepancy between the PRP aggregation experiment and the whole blood flow cytometry experiment could be explained by reduced potency of liraglutide in the presence of albumin and to aggregation being a less sensitive method to asses platelet function than flow cytometry. Our data suggest that liraglutide remains functional in whole blood and in the presence of albumin.

It is worth noting that the dose of liraglutide that persistently inhibited platelet function in our experiments was suprapharmacological at 50µg/mL. This is significantly higher than the 0.1 – 0.2µg/mL plasma level of liraglutide when given to patients at the normal therapeutic dose of 1.8mg od. It is reassuring that the toxicity study was negative and that the inhibitory effects of liraglutide 50µg/mL on aggregation in isolated platelet reversed at 90min incubation time and when platelets were stimulated with higher doses of the agonist. Although, using liraglutide at the pharmacological plasma level of 0.1 – 0.2µg/mL did not significantly affect platelet function in our experiments, this does not rule out an

effect for the drug on platelet function when given *in vivo*. This is because our experimental conditions and incubation times do not mimic normal physiology and it remains unclear how platelets will behave when exposed to this level of liraglutide in the blood for the duration of their lifespan. Although clinical outcome data in people with T2DM treated with liraglutide are lacking, animal studies suggest that liraglutide may have a cardio-protective effect. Noyan-Ashraf et al. (265) examined the outcome after experimental coronary artery occlusion in mice pretreated with the GLP-1R agonist liraglutide. Liraglutide, compared to saline, reduced cardiac rupture (12 of 60 versus 46 of 60; P=0.0001), infarct size (21±2% versus 29±3%, P=0.02) and improved cardiac output (12.4±0.6 versus 9.7±0.6 ml/min; P=0.002). The effects of liraglutide on survival were independent of weight loss. As platelets play a key role in atherothrombosis it is not known if part of this observation was due to a change in platelet function. Our data suggest that liraglutide may have a beneficial effect on platelet function and its effect on atherothrombotic risk in people with T2DM and obesity warrant further investigation.

Our work has many strengths including the use of well validated methods for the identification of a novel functional receptor in platelets and testing for the first time the effects of GLP-1R agonist, liraglutide, on human platelets. Limitations to our work include that only when using suprapharmacological doses of liraglutide was a significant effect on platelet function observed, which may undermine the clinical utility of our findings. In addition the mechanisms by which liraglutide inhibits platelets were not fully explained. However, our observations are preliminary and more studies are needed to confirm these findings and to establish the exact mechanism by which liraglutide works in platelets. Further studies may include examining the effects of liraglutide on; PKA substrates phosphorylation in the presence of PKA inhibitors; calcium concentration; AMPK phosphorylation; comparing the effects of liraglutide to native GLP-1; and conducting animal studies on wild and GLP-1R$^{-/-}$ mice. Our data highlight the need for clinical studies examining the CV outcome in people with T2DM and/or obesity treated with liraglutide.

3.7 Conclusions.

In summary, GLP-R is a novel receptor in platelets and its function and clinical effect are worth further evaluation. GLP-1R agonist, liraglutide, may have beneficial effects on platelet function.

4. Chapter 4: The effects of treatment with Liraglutide on athero-thrombotic risk in obese young women with polycystic ovary syndrome and controls.

4.1 Abstract.

Polycystic ovary syndrome (PCOS) is associated with obesity and increased cardiovascular (CV) risk markers. The objective of this study was to assess the effects of six months treatment with liraglutide 1.8mg od on obesity, and CV risk markers, particularly platelet function, in young obese women with PCOS compared to matched controls. We performed an interventional case-control study. Carotid intima-media wall thickness (cIMT) was measured by B-mode ultrasonography, platelet function by flow cytometry, clot structure/lysis by turbidimetric assays and endothelial function by ELISA and post-ischaemic reactive hyperemia (RHI). Data presented as mean change (6-month – baseline) ±standard deviation.

Nineteen obese women with PCOS and 17 controls, age 33.9 ±6.7 vs. 33.5 ±7.1 years and weight 102.1 ±17.1 vs. 100.4 ±15.1kg, respectively, were recruited; baseline atherothrombotic risk markers did not differ between the two groups. Twenty five (69.4%) participants completed the study (13 PCOS, 12 controls). At six months, weight was significantly reduced by 3.0 ±4.2 and 3.8 ±3.4kg in the PCOS and control groups, respectively; with no significant difference between the two groups, P=0.56. Similarly, HOMA-IR, triglyceride, hsCRP, urinary isoprostanes, serum endothelial adhesion markers (sP-selectin, sICAM and sVCAM), and clot lysis area were equally significantly reduced in both groups compared to baseline. Basal platelet P-selectin expression was significantly reduced at six months in controls -0.17 ±0.26 but not PCOS -0.12 ±0.28; between groups difference: 95% confidence interval= -0.14 – 0.26, P=0.41. No significant changes were noted in cIMT or RHI.

In conclusion, six months treatment with liraglutide 1.8mg od equally affected young obese women with PCOS and controls, and was associated with 3–4% weight loss and significant reduction in atherothrombosis markers including inflammation, endothelial function and clotting. Our data support the use of liraglutide as weight loss medication in simple obesity and suggest a potential beneficial effect on platelet function and atherothrombotic risk at 6 months of treatment.

4.2 Introduction.

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in women of reproductive age (5). Women with PCOS commonly present with oligomenorrhoea, hirsutism, subfertility and obesity (5). Obesity may play a role in the aetiology of PCOS and weight loss has been found to improve many clinical features of PCOS including menses regularity and fertility (69).

Despite the lack of long term cardiovascular (CV) outcome data, PCOS has been linked to several CV risk markers including type 2 diabetes (325), increased carotid intima-media wall thickness (cIMT) (347) and impaired platelet function (125, 191). We have reported recently that obesity, rather than the PCOS phenotype, appears to have the greatest impact on atherothrombotic risk parameters (348). Weight loss has been found to reduce many CV risk markers in PCOS including inflammation, and insulin resistance (IR) (349). Furthermore, in a recent study in healthy obese men and women, weight loss improved platelet function, by increasing platelets' sensitivity to the inhibitory effect of prostacyclin (PGI_2) and nitric oxide (NO) in aggregation (271). However, studies investigating the effect of weight loss on platelet function and cIMT in obese women with PCOS are lacking.

Lifestyle modifications and orlistat therapy are the only currently licensed treatments for obese women with PCOS not suitable for bariatric surgery. However, their efficacy is limited by the low compliance rate (91) and gastrointestinal side effects with orlistat. Recently, a glucagon like peptide-1 (GLP-1) mimetic, exenatide, has been found to improve weight and menses regularity in obese women with PCOS (113). Liraglutide is a long acting GLP-1 analogue with 97% resemblance to the native GLP-1 and seems to be better tolerated than exenatide, which has 53% resemblance to human GLP-1 (115). Similar to native GLP-1, it causes glucose dependent insulin secretion, promotes weight loss (114), and may subsequently improve IR. Although liraglutide is currently only licensed for the treatment of people with type 2 diabetes, it represents an attractive option for the treatment of obese women with PCOS.

The primary aim of this study was to assess whether six months treatment with liraglutide 1.8mg once a day (od) would have an impact on body weight, platelet function and cIMT in young obese women with PCOS compared to age and weight matched controls. Study secondary outcomes were to measure changes in anthropometric parameters, IR, lipids profile, inflammation, oxidative stress, clot structure/lysis, and endothelial function.

We hypothesised that women with PCOS and age and weight matched controls respond equally to treatment with liraglutide.

4.3 Study design.

Interventional case-control study of young obese women with PCOS and age and weight matched controls. The study was approved by the Leeds (East) Research Ethics Committee and an informed consent was given by all study participants.

4.4 Study participants.

The baseline characteristics of study participants and their selection prior to treatment have been described (348). Women with PCOS were recruited from the endocrine clinic and controls were recruited through an advertisement in the local newspaper. Subjects were invited for a screening visit if they had a body mass index (BMI) between 30–45 kg/m^2, were between 18–45 years of age and on no medications. PCOS was diagnosed according to the Rotterdam criteria (7). Participants with an alcohol intake of >14 units/week were also excluded. The metabolic syndrome was defined according to the International Diabetes Federation criteria 2006 (313). Participants who fulfilled the study inclusion/exclusion criteria were treated with liraglutide 0.6mg od subcutaneous injection for 1 week, 1.2mg od for one week and then 1.8mg od thereafter for six months. Study participants received no dietary advice. Participants were seen at baseline, three and six months after starting the treatment.

4.5 Methods.

A detailed description of the methods used for measuring anthropometric, biochemical markers, cIMT, platelet, endothelial and clotting functions have been

described in Chapter 2 and recently published (348). A brief description is outlined below.

4.5.1 Biochemical markers.

Venous blood samples were collected in the morning after a minimum of a 10h fast and samples were stored at -80ºC till batch analysis. Overnight urine samples were collected and aliquots stored at -20°C until batch analysis. Biochemical markers measured included high sensitivity C-reactive protein (hsCRP), testosterone, sex hormone binding globulin (SHBG), free androgen index (FAI), lipids profile, and urinary isoprostanes (8-iso $PGF_{2\alpha}$). Fasting plasma glucose (FPG) and insulin were measured to calculate the homeostatic model assessment of insulin resistance (HOMA-IR): FPG (mmol/L) X fasting insulin (iu/ml))/22.5 and β-cell function (HOMA-β): 20 X fasting insulin / (FPG – 3.5).

4.5.2 Carotid intima-media wall thickness (cIMT).

cIMT was measured using a Toshiba Xario 15 scanner (Toshiba Medical Systems, Tokyo, Japan) equipped with 11-MHz linear imaging probe. The cIMT measurements were performed in accordance with recommendations by the Mannheim consensus, 2006 (131). All measurements were performed by the same trained operator. Reproducibility of cIMT measurements was assessed in 18 participants who underwent 2 ultrasound examinations within a 4 weeks period using the Bland Altman plot; mean difference=-0.002 (95% limits of agreement=-0.055, 0.059); intraclass correlation coefficient (ICC)=0.95 (95% CI=0.90, 0.99).

4.5.3 Platelet function.

Fluorescein isothiocyanate (FITC)-conjugated anti human CD42b antibody, phycoerythrin (PE)-conjugated anti human CD62P, FITC-anti IgG_{1k} and PE-anti IgG_{1k} isotope controls were obtained from BD bioscience (Oxford, UK). FITC-anti human fibrinogen was obtained from Dako (Stockport, UK). Prostacyclin I_2 (PGI_2) was obtained from Caymen (USA) and Adenosine 5'-diphosphate (ADP) from Sigma (Poole, UK).

Platelet function was analysed in whole blood by flow cytometry according to the methods described by Goodall et al. (292). The platelet population was identified by

their forward and side-scatter characteristics and confirmed by the expression of platelet-specific surface marker CD42b. P-selectin surface expression, a marker of platelet α-granule content release, and fibrinogen binding, a marker of platelet activation, were calculated from 10,000 platelet events. In brief, 5µl of citrated blood was diluted in 50µl of modified Tyrodes buffer (150mM NaCl, 5mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], 0.55mM NaH2PO4, 7mM NaHCO3, 2.7mM KCl, 0.5mM MgCl2, 5.6mM glucose, pH 7.4) and mixed with 2µL of the appropriate FITC or PE labelled antibody. Samples were fixed with 500µl of 0.2% paraformaldehyde and analysed by flow cytometry (BD FACSAria). In some cases, samples were incubated with ADP in the presence and absence of PGI_2 before fixation.

4.5.4 Endothelial function.

Endothelial function was examined by measuring the post-ischaemic reactive hyperaemic index (RHI) using Endo-PAT2000 (Itamar Medical Ltd, Israel) (297). The serum concentrations of endothelial adhesion markers sP-selectin, intercellular adhesion molecule-1 (sICAM-1), and vascular cell adhesion molecule-1 (sVCAM-1) were measured by ELISA as per manufacturer's protocol.

4.5.5 Clot formation/lysis.

Clot structure analysis was measured using turbidimetric analysis as previously described (350). The clot parameters assessed included lag phase, maximum absorbance (MA), lysis time (LT), clot formation time and lysis area (LA). Higher MA of plasma clots, longer LT and larger LA are associated increased CV risk (311).

4.5.6 Power calculations and statistical analysis.

The sample size for platelet function was powered using the Mann-Whitney U test for continuous data (314) and an 'effect size' between 1.12–1.25 for changes in P-selectin expression in unstimulated samples (187, 315). A sample size of 18 per group allow us to detect an effect size of 1.12 (or larger) with 80% power, 20% attrition and 5% significance (two-tailed) between cases and controls. As variations within groups are usually smaller than between groups, then this study was adequately powered to detect differences' pre/post treatment.

Data were summarised by the mean and SD (continuous data) or by percentages (categorical data). Data were checked for normality using Kolmogorov-Smirnov test. Differences between groups at baseline were analysed using the independent t-test for continuous data (or Mann-Whitney U test for non-normally distributed data). Frequency distributions' were analysed using Chi-square test. Between groups' comparisons after intervention were as follows: for each group (PCOS and controls) a difference between baseline and 6 months was calculated. The between group differences were compared using the independent t-test (or Mann-Whitney U test for non-normally distributed data).

Within groups analysis (baseline vs. six months) was performed using the dependent t-test for continuous data (or Wilcoxon signed-rank test for non-normally distributed data). Data were analysed using intention to treat analysis with missing values for sequential data imputed by carrying the last observation forward (351). Statistical analysis was performed using the PASW statistics 19 package (SPSS Inc., Chicago, USA). A two tailed P value of <0.05 was considered statistically significant.

4.6 Results.

4.6.1 Baseline characteristics.

Fifty one women were screened, 36 recruited (19 PCOS, 17 normal controls). Fifteen women were excluded as they had; idiopathic hirsutism (five), hypothyroidism (one), BMI <30kg/m^2 (one), no trans-vaginal ultrasound (TV USS) scan was done (two), ovaries not visible on TV USS (one), oligomenorrhoea without PCOS (one), and repeatedly missing the initial study visit (four).

The PCOS and control groups were matched for age 33.9 ±6.7 vs. 33.5 ±7.1 years, weight 102.1 ±17.1 vs. 100.4 ±15.1kg, BMI 37.9 ±5.0 vs. 36.5 ±4.6 kg/m^2, and waist 112.7 ±12.6 vs. 112.4±9.4 cm, respectively. The PCOS group had higher testosterone 1.3 ±0.4 vs. 0.9 ±0.3nmol/L (P=0.01), FAI 4.4 ±2.0 vs. 2.6 ±1.2 (P=0.01), fasting insulin 22.0 ±9.4 vs. 16.1 ±5.6 iu/ml (P=0.03) and HOMA-IR 5.1 ±2.6 vs. 3.5 ±1.3 (P=0.03) compared to controls at baseline. cIMT, platelet function, clot

formation/lysis, and endothelial function did not significantly differ between the two groups at baseline as described in Chapter 2.

4.6.2 Intervention with liraglutide.

Twenty five women, 69%, completed the study (13 PCOS, and 12 controls). Eleven women dropped out because of: nausea and vomiting (four), loss of follow up (four), frequently missing study drug (one), change in personal circumstances (one), and pregnancy (one).

4.6.3 Demographics.

Following 6 months of treatment with liraglutide 1.8mg od body weight was reduced by 3.0 ±4.2 (P<0.01) and 3.8 ±3.4kg (P<0.01); BMI by 1.0 ±1.5 (P=0.01) and 1.4 ±1.2kg/m^2 (P<0.01); while average heart rate increased by 3 ±7 (P=0.051) and 4 ±7 beat per minute (bpm) (P=0.02) in the PCOS and control groups, respectively. There was no significant change in waist circumference, systolic and diastolic blood pressure, or in the prevalence of the metabolic syndrome (Table 4.1). Between groups comparisons were not significant (Table 4.2).

4.6.4 Biochemical profile.

At 6 months FPG, fasting insulin, HOMA-IR, hsCRP, triglycerides, and urinary isoprostane significantly reduced in both groups. There was no significant change in other biochemical markers including testosterone, SHBG, FAI, HOMA-β or cholesterol (Table 4.1). Between groups comparisons were not significant (Table 4.2).

4.6.5 cIMT.

There was no significant change in average cIMT at baseline compared to 6 months; 0.51 ±0.05 vs. 0.51 ±0.06mm (P=0.72) and 0.48 ±0.06 vs. 0.48 ±0.06mm (P=0.35) in the PCOS and control groups, respectively. Between groups comparisons were not significant (Table 4.2).

	PCOS (n=19)			Controls (n=17)		
	Baseline	**6m**	**P**	**Baseline**	**6m**	**P**
Weight (kg)	102.1 ±17.1	99.1 ±15.9	<0.01	100.4 ±15.1	96.7 ±14.4	<0.01
BMI (kg/m^2)	37.9 ±5.0	36.9 ±4.8	0.01	36.5 ±4.6	35.1 ±4.3	<0.01
Waist (cm)	112.0 ±12.6	110.9 ±12.4	0.31	112.3 ± 9.7	110.9 ±8.8	0.12
Systolic BP (mmHg)	121 ±13.0	121 ±10.5	1.0	126 ±12.9	121 ±14.1	0.085
Diastolic BP (mmHg)	75 ±7.6	76 ±7.9	0.91	79 ±6.6	75 ±7.8	0.088
Average HR (bpm)	67.7 ±7.7	71.2 ±9.7	0.051	67.3 ±9.0	71.4 ±7.0	0.018
Metabolic syndrome	10 (52.6%)	6 (31.6%)	0.19	6 (35.3%)	6 (35.3%)	1
Testosterone(nmol/L)	1.3 ±0.4	1.2 ±0.5	0.78	0.90 ±0.3	0.94 ±0.2	0.63
FAI	4.4 ±2.0	4.4 ±2.7	0.86	2.6 ±1.2	2.5 ±0.8	0.78
SHBG (nmol/L)	33.2 ±14.6	30.8 ±10	0.29	38.1 ±14.4	40.3 ±12.7	0.29
FPG (mmol/L)	5.1 ±0.6	4.9 ±0.7	0.045	4.9 ±0.4	4.5 ±0.4	<0.01
Insulin (iu/ml)	22.0 ±9.4	19.5 ±9.0	0.025	16.1 ±5.6	12.8 ±4.9	<0.01
HOMA-IR	5.1 ±2.6	4.3 ±2.4	0.012	3.5 ±1.3	2.6 ±1.1	<0.01
HOMA-β	303 ±135	314 ±169	0.63	255 ±114	266 ±107	0.69
hsCRP (mg/L)	6.2 ±8.7	4.3 ±3.9	0.025	5.1 ±3.4	3.9 ±3.0	0.038
Total chol (mmol/L)	5.0 ±0.8	4.9 ±0.8	0.24	5.1 ±1.0	5.1 ±0.9	0.88
LDL (mmol/L)	3.2 ±0.6	3.2 ±0.6	0.38	3.3 ±0.9	3.4 ±0.8	0.51
HDL (mmol/L)	1.2 ±0.2	1.2 ±0.3	0.59	1.3 ±0.2	1.2 ±0.2	0.55
Tri (mmol/L)	1.4 ±0.7	1.1 ±0.5	<0.01	1.4 ±0.7	1.1 ±0.6	0.027
Chol/HDL	4.4 ±0.9	4.3 ±0.7	0.83	4.1 ±0.8	4.3 ±1.1	0.43
Isoprostane (ng/ml)	0.81 ±0.53	0.36 ±0.48	0.01	0.58 ±0.57	0.21 ±0.19	0.031
Fibrinogen (mg/ml)	3.8 ±1.2	3.8 ±1.0	0.83	3.5 ±1.0	3.5 ±0.7	0.72
RHI	1.9 ±0.5	1.9 ±0.5	0.96	1.9 ±0.5	2.0 ±0.6	0.5
sP-selectin (ng/ml)	135 ±40.3	99.5 ±49	<0.01	126.4 ±25.5	79.4 ±39.2	<0.01
sICAM-1 (ng/ml)	302 ±53.3	249 ±66.4	<0.01	302.1 ±36.9	223.8±44.4	<0.01
sVCAM-1 (ng/ml)	3384 ±379	1784±1022	<0.01	3449 ±404	1625 ±675	<0.01

Table 4.1 Within group comparisons of anthropometric, metabolic and biochemical parameters at baseline and after treatment with liraglutide. BMI, body mass index; BP, blood pressure; HR, heart rate; bpm, beat per minute; FAI, free androgen index; SHBG, sex hormone binding globulin; FPG, fasting plasma glucose; HOMA-IR, homeostatic model assessment-insulin resistance; HOMA-β, homeostatic model assessment-β cell function; hsCRP, high sensitivity C-reactive protein; LDL, low density lipoprotein; HDL, high density lipoprotein; Tri, triglycerides; Chol, cholesterol; RHI, reactive hyperemic index; sICAM, soluble intercellular adhesion molecule-1; sVCAM, soluble vascular cell adhesion molecule-1. Data are mean ±SD (standard deviation) or number (percentage). P-values are for within group comparisons, baseline vs. 6-months.

4.6.6 Platelet function.

Following 6 months treatment with liraglutide there was significant inhibition of P-selectin expression in unstimulated samples in the control group, compared to baseline, without change in fibrinogen binding (Table 4.3). P-selectin expression and fibrinogen binding levels were unchanged in the PCOS group at 6 months compared to baseline. Similarly, platelet response to activation by ADP (0.1-10µM) or to inhibition by PGI_2 (1-100nM) were unchanged at 6 months in either group (Table 4.3, Figure 4.1). Between groups comparisons were not significant (Table 4.4).

4.6.7 Clot function/lysis.

At six months, clot lag phase significantly increased and LA reduced in both groups compared to baseline (Figure 4.2). However, other markers did not significantly change in either group including clot LT, clot formation time, and clot MA (Figure 4.2). There was no significant change in fibrinogen levels at 6 months compared to baseline (Table 4.1). Between groups comparisons were not significant (Table 4.4).

4.6.8 Endothelial function.

There was no significant change in RHI, at six months compared to baseline, in either group (Table 4.1). Serum markers of endothelial function sP-selectin, sVCAM-1 and sICAM-1 significantly reduced after treatment in both groups (Table 4.1). Between groups comparisons were not significant (Table 4.2).

Variable	PCOS 6m – baseline	Controls 6m – baseline	Between groups difference	
			95%CI	P-value
Weight (kg)	-3.0	-3.8	-1.9 – 3.4	0.56
BMI (kg/m^2)	-1.0	-1.4	-0.6 – 1.3	0.43
Waist (cm)	-1.1	-1.4	-2.4 – 3.2	0.78
Systolic BP (mmHg)	0.0	-4.4	-2.9 – 11.7	0.23
Diastolic BP (mmHg)	0.3	-3.9	-2.1 – 10.4	0.19
Average HR (bpm)	3.0	4.0	-5.0 – 4.0	0.77
Testosterone (nmol/L)	-0.03	0.04	-0.4 – 0.2	0.63
FAI	0.09	-0.06	-1.2 – 1.5	0.76
SHBG (nmol/L)	-2.4	2.3	-10.9 – 1.7	0.50
FPG (mmol/L)	-0.2	-0.3	-0.2 – 0.4	0.40
Insulin (iu/ml)	-2.6	-3.4	-2.3 – 3.9	0.61
HOMA-IR	-0.8	-0.9	-0.6 – 0.9	0.70
HOMA-β	11.2	10.7	-70.0 – 70.9	1
hsCRP (mg/L)	-1.9	-1.2	-4.3 – 2.9	0.21
Total chol (mmol/L)	-0.1	-0.02	-0.4 – 0.3	0.64
LDL (mmol/L)	0.06	0.08	-0.3 – 0.3	0.89
HDL (mmol/L)	-0.02	-0.04	-0.1 – 0.2	0.84
Tri (mmol/L)	-0.3	-0.2	-0.4 – 0.2	0.56
Chol/HDL	-0.03	0.1	-0.6 – 0.3	0.37
Isoprostane (ng/ml)	-0.5	-0.4	-0.5 – 0.3	0.64
Fibrinogen (mg/ml)	0.02	-0.03	-0.4 – 0.5	0.84
cIMT	0.002	0.004	-0.01 – 0.02	0.41
RHI	-0.01	0.1	-0.8 – 0.6	0.68
sP-selectin (ng/ml)	-36.4	-47.1	-13.7 – 35.1	0.38
sICAM-1 (ng/ml)	-53.6	-78.2	-16.8 – 66.0	0.24
sVCAM-1 (ng/ml)	-1600	-1824	-335 - 782	0.42

Table 4.2 Between groups comparison for changes in anthropometric, metabolic and biochemical parameters after treatment with liraglutide. Data presented as mean change (6-months – baseline). 95%CI, 95% confidence interval; BMI, body mass index; BP, blood pressure; HR, heart rate; bpm, beat per minute; FAI, free androgen index; SHBG, sex hormone binding globulin; FPG, fasting plasma glucose; HOMA-IR, homeostatic model assessment-insulin resistance; HOMA-β, homeostatic model assessment-β cell function; hsCRP, high sensitivity C-reactive protein; LDL, low density lipoprotein; HDL, high density lipoprotein; Tri, triglycerides; Chol, cholesterol; cIMT, carotid intima-media wall thickness; RHI, reactive hyperemic index; sICAM, soluble intercellular adhesion molecule-1; sVCAM, soluble vascular cell adhesion molecule-1.

	PCOS			Controls		
	Baseline	**6 months**	**P**	**Baseline**	**6 months**	**P**
P-selectin						
Basal	0.52 ±0.3	0.40 ±0.3	0.12	0.42 ±0.2	0.24 ±0.16	0.02
ADP 0.1µM	3.1 ±2.9	3.0 ±2.1	0.82	2.8 ±2.1	2.7 ±2.5	0.83
ADP 1µM	40.2 ±14.5	40.4 ±17.4	0.9	44.0 ±13.5	42.9 ±13.1	0.67
ADP 10µM	61.6 ±13.6	61.5 ±14.6	0.97	66.9 ±13.1	69.8 ±10.5	0.22
Fibrinogen binding						
Basal	0.97 ±0.4	0.93 ±0.3	0.67	0.83 ±0.3	0.92 ±0.4	0.28
ADP 0.1µM	9.7 ±10.0	11.2 ±9.6	0.31	7.7 ±7.1	5.1 ±4.0	0.11
ADP 1µM	55.9 ±18.4	56.4 ±21.7	0.79	55.8 ±19.0	51.2 ±13.5	0.22
ADP 10µM	73.7 ±14.6	74.8 ±15	0.61	76.2 ±11.7	72.2 ±9.8	0.08

Table 4.3 The effects of six months treatment with liraglutide on platelet activation. Platelet surface expression (% of positive cells) of P-selectin and fibrinogen binding were measured in unstimulated samples (basal activation) and after stimulation with ADP 0.1-10µM for 20 minutes before fixing with 0.2% paraformaldehyde. Data are presented as mean ±SD (standard deviation). ADP, adenosine-5'-diphosphate.

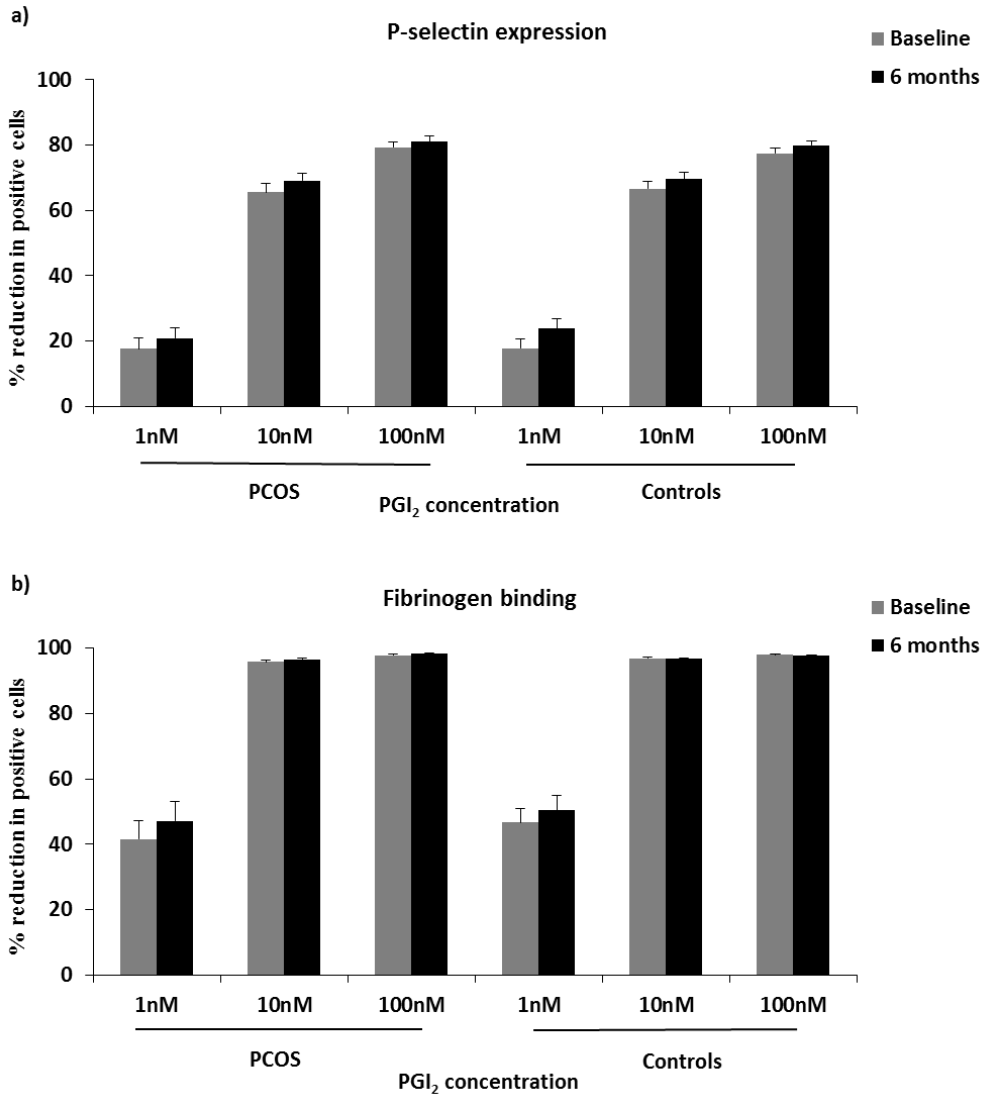


Figure 4.1 The effects of six months treatment with liraglutide on platelet sensitivity to the inhibitory effect of prostacyclin. Samples were pre incubated with PGI2 1, 10, and 100nM for 90 seconds before; stimulation with ADP 10µM and fixing at 20 minutes for P-selectin expression (Fig 2a); or stimulation with ADP 1µM and fixing at 5 minutes for fibrinogen binding (Fig 2b). Data are presented as mean ±SEM (standard error of the mean). ADP, adenosine-5'-diphosphate; PGI2, prostacyclin. All P values were >0.05.

Variable	PCOS 6m – baseline	Controls 6m – baseline	Between groups difference	
			95%CI	P-value
Platelet P-selectin expression				
Basal activation	-0.12	-0.17	-0.14 – 0.26	0.41
ADP 0.1µM	-0.09	-0.16	-1.6 – 1.7	0.42
ADP 1µM	0.2	-1.1	-4.8 – 7.3	0.74
ADP 10µM	-0.1	2.9	-8.7 – 2.8	0.30
% inhibition with PGI_2:				
1nM	3.4	6.0	-9.8 – 4.6	0.80
10nM	3.5	3.0	-7.1 – 8.1	0.67
100nM	1.7	2.5	-5.6 – 4.0	0.74
Platelet fibrinogen binding				
Basal activation	-0.04	0.09	-0.4 – 0.13	0.31
ADP 0.1µM	1.6	-2.6	-0.06 – 8.4	0.13
ADP 1µM	0.6	-4.6	-3.4 – 13.8	0.30
ADP 10µM	1.1	-4.0	-0.95 – 11.2	0.10
% inhibition with PGI_2:				
1nM	5.6	3.6	-13.7 – 17.6	0.90
10nM	0.7	0.1	-0.9 – 2.5	0.28
100nM	0.5	0.2	-0.4 – 1.7	0.21
Clot function/lysis				
Lag phase (sec)	96	139	-99 – 12.8	0.13
Lysis area (au)	-108	-100	-139 – 124	0.59
Maximum absorbance (au)	-0.01	-0.03	-0.03 – 0.06	0.49
Clot formation time (sec)	69	40	-83 – 140	0.61
Lysis time (sec)	7.1	20.9	-136 – 108	0.82

Table 4.4 Between groups comparisons for changes in platelet function and clot function/lysis after treatment with liraglutide. Platelet surface expression of P-selectin and fibrinogen binding were measured in unstimulated samples and after stimulation with ADP 0.1-10µM for 20 minutes before fixing with 0.2% paraformaldehyde. For platelet sensitivity to PGI_2, samples were pre incubated with PGI_2 1-100nM for 90 seconds before; stimulation with ADP 10µM and fixing at 20 minutes for P-selectin expression; or stimulation with ADP 1µM and fixing at 5 minutes for fibrinogen binding. Data are mean change (6-months – baseline); 95%CI, 95% confidence interval; ADP, adenosine-5'-diphosphate; PGI_2, prostacyclin; sec, seconds; au, arbitrary unit.

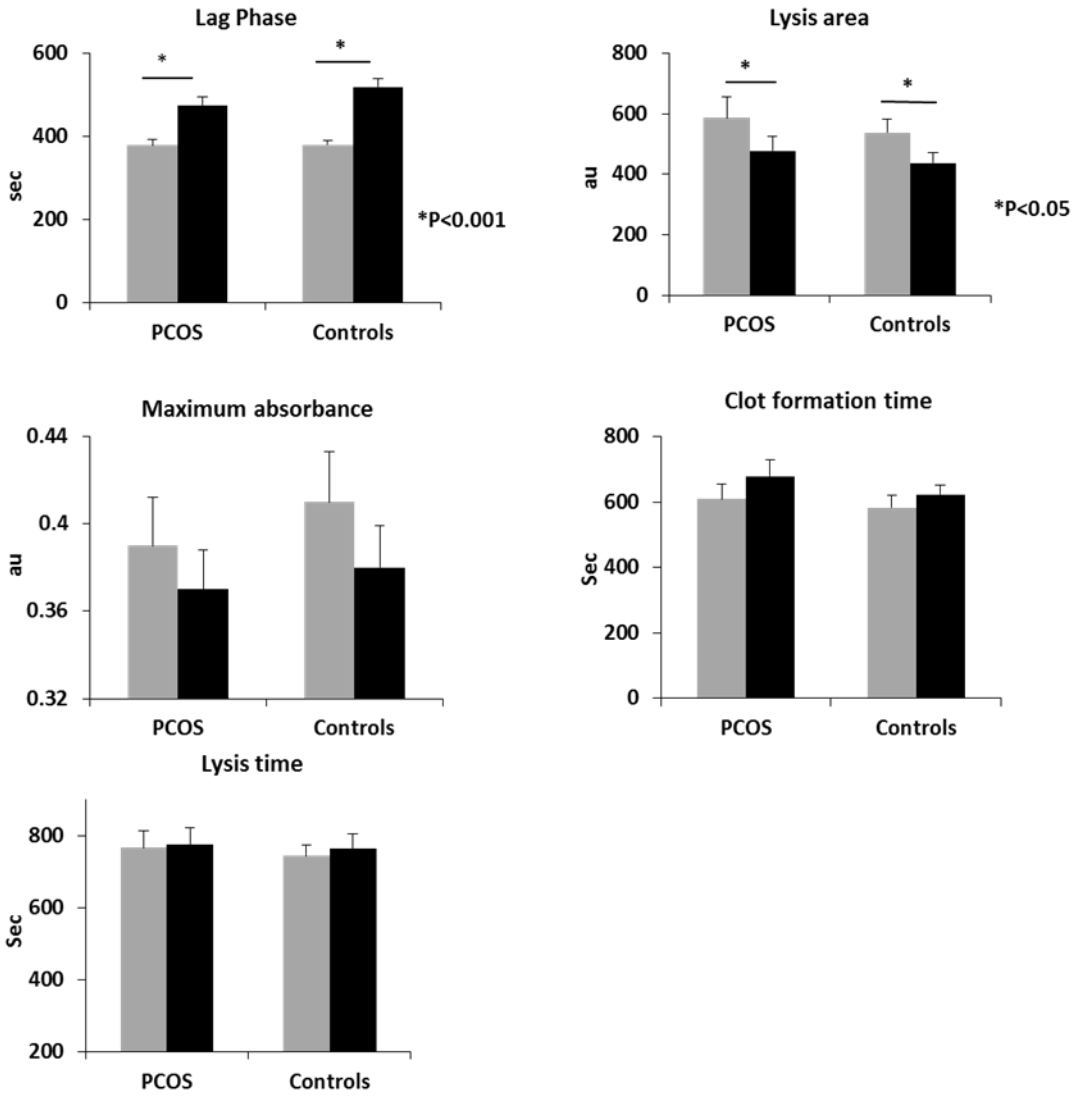


Figure 4.2 The effects of six months treatment with liraglutide on clot function/lysis. Data presented as mean ±SEM (standard error of the mean). Baseline data are represented in grey colour, and 6 months data are in black. sec, seconds; au, arbitrary units.

4.7 Discussion.

Our data suggest that young obese women with PCOS and weight matched controls respond equally to treatment with liraglutide. Six months treatment with liraglutide 1.8mg od resulted in a small, though significant, 3 – 4% degree of weight loss that was associated with significant reductions in IR, inflammation, oxidative stress and improvement in several CV risk markers in young obese women with and without PCOS. Our findings are important as the majority of previous reports suggest the

need for moderate weight loss (≥10%) to achieve reduction in atherothrombotic risk (193, 271).

The weight loss of 3 – 4% achieved in our study with liraglutide 1.8mg od is in accord with other published data (114), and although a higher dose of liraglutide might have resulted in more pronounced weight loss (194), we have chosen the highest dose currently licensed for the treatment of people with type 2 diabetes (264); a dose with well-known safety profile and adverse reactions. The effects of liraglutide on weight loss are thought to be mainly mediated through delayed gastric emptying and reduced appetite, rather than a change in energy expenditure (352). While its effects on glucose metabolism are secondary to an increase in insulin secretion in a glucose-dependent manner, suppression of glucagon secretion, enhanced hepatic insulin action, and reduced β-cell apoptosis (344, 353, 354). Similar to native GLP-1, liraglutide is widely believed to exert its actions through the GLP-1 receptor (GLP-1R) and the activation of cyclic adenosine monophosphate (cAMP) dependent pathway (264). This is supported by the wide expression of GLP-1R including in the pancreas, stomach, and brain (354). However, the underlying mechanisms for GLP-1 mediated weight loss remain poorly understood and may involve direct, GLP-1R mediated, and indirect, e.g. neuronal, pathways (354).

This is the first study to examine the impact of treatment with GLP-1 analogues on platelet function in obese women with or without PCOS. In this study, there was a significant inhibition in basal platelet P-selectin expression after treatment with liraglutide in the control group only. P-selectin is only expressed on the platelet surface membrane after *α* granule secretion (i.e. platelet activation) (289). It mediates adhesion of activated platelets to neutrophils and monocytes (290); plays a central role in platelet aggregate size and stability (355), and may play an important role in the pathogenesis of inflammation and thrombosis (355). Subsequently, a decrease in P-selectin expression on platelet surface after treatment with liraglutide may predict a favourable CV outcome. We did not find improvement in platelet sensitivity to PGI_2, which was reported after diet-induced weight loss in simple obesity (271), though this may reflect the modest weight loss

achieved (3-4%). Weight loss has also been found to reduce urinary thromboxane A_2 metabolite excretion and improve platelet function in obese women over a three months period (193). The improvement in platelet function with weight loss was related to a reduction in abdominal obesity and associated reduction in IR, inflammation and oxidative stress levels (193, 271). Platelets have been found to express the insulin receptor (356) and while insulin is believed to reduce platelet sensitivity to aggregating agents, its function in IR states for example type 2 diabetes and obesity is thought to be impaired (357). As PCOS, similar to type 2 diabetes, is associated with increased IR independent of obesity, it is possible that platelets from women with PCOS are more resistant to the inhibitory effects of liraglutide and/or associated weight loss, perhaps suggesting an inherent defect in platelet function in PCOS. However, it is worth noting that between group comparisons were not significant and it is so possible that the lack of significant change in basal platelet P-selectin expression in the PCOS group after treatment is related to higher than anticipated dropout during the study. In addition, we have found in the previous chapter that platelets express the GLP-1R; subsequently the changes in platelet function noted in our study may represent an independent effect of liraglutide.

There was a significant reduction in clot lysis area, a complex measure of clot formation, density and lysis potential, after six months treatment with liraglutide in both groups. The majority of previous studies examining clot function and fibrinolysis in obesity reported a reduction in fibrinogen levels and reduction in plasminogen activator-1 (PAI-1) activity after ≥ 5 – 10% weight loss (358). Interestingly, fibrinogen levels did not significantly change in our study after treatment suggesting that the changes in clot structure parameters observed were probably related to other plasma proteins, yet to be identified; an independent effect of liraglutide; or to qualitative changes in fibrinogen induced by weight loss.

Treatment with liraglutide did not change cIMT measurement in either group despite significant reduction in IR. This is the converse to what was reported by Orio et al. (152) who treated 30 lean women with PCOS with metformin for six months and found a significant reduction in cIMT related to an improvement in IR.

In another study (359), weight loss of 3.9 kg/m^2 over one year of dietary and lifestyle intervention in obese adolescent girls with PCOS resulted in a significant reduction in cIMT. The change in BMI in our study was smaller, 1.0 – 1.4 kg/m^2, the follow up duration was shorter, and no diet and/or exercise interventions were included which may account for the discrepancy.

Endothelial function measured by post-ischaemic RHI was unchanged following liraglutide therapy that was discordant to the significant reduction in the endothelial serum adhesion markers sP-selectin, sVCAM, and sICAM. This is in accord with other studies showing that changes in clinical and serum markers of endothelial function in response to moderate weight loss do not always correlate. Keogh et al. reported significant improvement in sP-selectin and sICAM-1 but no change in FMD at 8 weeks despite 5-10% weight loss (360). In another study, no change in FMD was noted despite 4 – 8% weight loss in a large cohort of obese subjects over two year period (361). Conversely, weight loss was associated with a reduction in cIMT and improvement in FMD at 18 months and 5 years of follow up following bariatric surgery (362). This suggests that serum markers of endothelial function are probably more sensitive to small changes in body weight, inflammation and oxidative stress that do not reflect overt functional endothelial changes.

Although treatment with liraglutide, in our study, has resulted in significant reduction in fasting plasma glucose, insulin, and insulin resistance (HOMA-IR), there was no improvement in beta cell function (HOMA-β). Preclinical studies suggest that liraglutide treatment increases beta cells mass (363). Treatment with liraglutide in a 20 week trial increased HOMA-β by 5 – 24% in obese men and women (114). The study included more participants than ours, 90 per group, which may explain the different results. Interestingly, there was no association between the dose of liraglutide and the change in β-cell function after treatment, median increase by 27.8% for liraglutide 1.8mg od and 8.4% for 2.4mg od, nor did the changes in HOMA-β correlate with HOMA-IR (which did not change during the study) (114). It is worth noting that HOMA-β, although simple and commonly used, has many limitations including the use of a fasting parameter of β-cell function,

which reflects basal rather than glucose-stimulated insulin secretion, and is also affected by alterations in insulin clearance (12, 364, 365).

Our data suggest a potential CV benefit for obese women treated with liraglutide; though how much of the change observed during the study was related to the effects of liraglutide *per se* or to the associated weight loss remains unclear and requires clarification. Ethical considerations precluded performing the clinical trial on non-obese subjects due to the liraglutide side effect profile. Ideally we would have included a diet only control group aiming to achieve the same amount of weight loss at 3 months. Animal studies suggest that liraglutide may have a cardioprotective effect independent of weight loss (265).

The strengths of this study include having obese women with PCOS and controls who were well matched for age, weight, BMI and waist circumference; the use of well-established markers to assess atherothrombotic risk; and it being the first study to assess the effects of treatment with a GLP-1 analogue on platelet function in obese women with and without PCOS. The main limitation to our study was the higher than anticipated dropout during the study, due to liraglutide's side effects, which may have compromised the study power. We are aware that interpretation of non-significant findings is challenging. Another limitation is the absence of a placebo-treated group, with equal amount of weight loss achieved, to clarify if the changes observed were related to liraglutide *per se* or to the associated weight loss. However, the results of this study would now allow a larger study to be powered appropriately to include a placebo.

4.8 Conclusions.

Six months treatment with liraglutide equally affected young obese women with PCOS and controls, and resulted in mild degree of weight loss (3 – 4%) and significant reduction in IR, inflammation, and oxidative stress. Several markers of atherothrombosis also improved including markers of endothelial function and clot structure, but no change in cIMT was observed. While basal platelet activation was only reduced in the control group, the between groups difference was not significant. Our data support the use of liraglutide as a weight loss medication in

simple obesity and suggest a potential beneficial effect on platelet function and atherothrombotic risk at 6 months of treatment.

5. Chapter 5: Glucagon like peptide-1 analogue, liraglutide, improves liver fibrosis markers in obese women with polycystic ovary syndrome and non-alcoholic fatty liver disease.

5.1 Abstract.

Non-alcoholic fatty liver disease (NAFLD) has been linked to polycystic ovary syndrome (PCOS) and carries an increased risk of liver cirrhosis. Procollagen type 3 amino-terminal peptide (PIIINP) is an independent predictor of liver cirrhosis. The aim of this study was to assess whether six months of treatment with the GLP-1 analogue, liraglutide, improves markers of liver fibrosis. We performed a case-controlled study comparing these markers in women with PCOS to age- and weight-matched controls. PCOS was diagnosed according to the Rotterdam criteria. All participants underwent liver function tests and a liver ultrasound scan to assess fatty infiltration. The serum marker of liver fibrosis, PIIINP, was measured at baseline and after six months treatment with liraglutide 1.8mg od.

Nineteen women with PCOS and 17 controls were recruited, age 32.8±7.2 vs. 33.5±6.7years and weight 100.9±16.7 vs. 99.3±14.7kg, respectively. At baseline, the PCOS group had higher testosterone 1.2±0.3 vs. 0.9±0.3nmol/L (P=0.01), HOMA-IR 5.1±2.6 vs. 3.5±1.3 (P=0.03), AST 22.4±5.2 vs.18.8±3.4u/L (P=0.04), PIIINP 4.4±0.8 vs. 3.5±0.8ug/ml (P=0.01) and NAFLD seven (35%) vs. none (P=0.005), respectively. Twenty five (69%) participants completed the study (13 PCOS, 12 controls). Following treatment, weight was reduced by 3.0±4.2kg (P=0.01) and 3.8±3.4kg (P=0.001), respectively. Similarly, HOMA-IR, hsCRP, triglycerides and urinary isoprostane significantly reduced in both groups. PIIINP significantly reduced the in PCOS group 4.4±0.8 vs. 3.7±0.9ug/ml (P<0.01), but not in controls 3.5±0.8 vs. 3.2±0.7ug/ml (P=0.08).

In conclusion, treatment with liraglutide, and/or associated weight loss, significantly reduced PIIINP levels in obese women with PCOS. This may be an additional beneficial factor when considering the use of liraglutide in women with PCOS, obesity and NAFLD.

5.2 Introduction.

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in women of reproductive age (5). Women with PCOS are insulin resistant and commonly present with oligomenorrhoea, clinical and/or biochemical hyperandrogensim and obesity (5, 75). Women with PCOS are at increased risk of the metabolic syndrome and its hepatic manifestation, non-alcoholic fatty liver disease (NAFLD) (366). Obesity, probably through fatty infiltration, is associated with elevated liver enzymes, NAFLD, cirrhosis and hepatocellular carcinoma (367-369). In addition, NAFLD itself carries an increased risk of developing non-alcoholic steatohepatitis (NASH), with subsequent increased risk of liver cirrhosis and liver-related mortality (370, 371). NAFLD is also associated with type 2 diabetes and has recently been proposed as a cardiovascular risk marker (372). Considering that early stages of hepatic steatosis, steatohepatitis and possibly fibrosis are reversible (366, 373, 374), early identification and treatment of NAFLD is important.

Hyaluronic acid (HA) and procollagen type III amino terminal peptide (PIIINP) are biochemical markers which have been suggested as independent predictors of liver cirrhosis in patients with chronic viral hepatitis (375). HA is a glycosaminoglycan which is a component of the liver extracellular matrix; it is produced by myofibroblasts and degraded by liver sinusoidal cells (376). PIIINP is a peptide which is cleaved from procollagen III during its excretion by fibroblasts; PIIINP levels are increased during liver fibrosis as collagen III accumulates in the liver extracellular matrix (376). In addition, PIIINP is found to discriminate between simple steatosis and NASH or advanced fibrosis in patients with NAFLD (377) and it is considered an early marker of liver fibrosis (378). Although it has been suggested that women with PCOS may benefit from earlier screening for liver disease (366), studies investigating methods to reduce the risk of NAFLDs and liver fibrosis in obese women with PCOS are sparse (379).

Liraglutide is a long acting glucagon like peptide-1 (GLP-1) analogue with 97% resemblance to the native GLP-1. Similar to native GLP-1, it causes glucose dependent insulin secretion (380), promotes weight loss (114), and may subsequently improve insulin resistance (IR). In addition, liraglutide has been found

to reduce hepatic steatosis in pre-clinical trials (381). Although it is currently only licensed for the treatment of people with type 2 diabetes (T2DM), it represents an attractive option for the treatment of obese women with PCOS.

The primary aim of this study was to investigate the effects of 6 months treatment with liraglutide on metabolic and liver disease markers in obese women with PCOS and controls. We hypothesised that liraglutide, and/or associated weight loss, would improve markers of liver disease in obese women with PCOS.

5.3 Study design.

A case-control study of young obese women with PCOS and age and weight matched controls. The study was approved by a local Ethics Committee and all study participants signed an informed consent form before participation.

5.4 Study participants and methods.

Women with PCOS and controls were invited for a screening visit if they had a body mass index (BMI) between 30–45 kg/m^2, were between 18–45 years of age and on no medications. PCOS was diagnosed according to the Rotterdam criteria (7). Other endocrine disorders with similar presentation were excluded. Control subjects underwent the same biochemical screening as the PCOS group to exclude any unknown endocrine disorder. Control subjects with a history of clinical or biochemical hirsutism or menstrual irregularities were excluded.

The metabolic syndrome was defined according to the International Diabetes Federation criteria 2006 (313). Liver ultrasound scan was performed, using Toshiba Xario 15 scanner, to investigate for evidence of fatty infiltration; with all scans performed by the same trained operator for consistency. Other causes of liver disease were excluded by measuring serum alpha-1 antitrypsin, ceruloplasmin, ferritin, autoimmune profile and hepatitis B and C serology. Participants with alcohol intake of >14 units/week were excluded from the study.

Participants, PCOS and controls, who fulfilled the study inclusion/exclusion criteria were treated with Liraglutide 0.6 mg od for one week, 1.2mg for one week and then 1.8mg od for six months. Study participants did not receive any special dietary

advice and were not advised to change their diet. Anthropometric, biochemical, and hormonal markers including, fasting insulin and glucose, HOMA-IR, hsCRP, were measured as described before (348). NAFLD clinical score (BARD) (382), and fibrosis (F) score (383) were measured at baseline. Serum markers of liver fibrosis, PIIINP and HA, were measured in the fasting state at baseline, 3, and 6 months after starting liraglutide. PIIINP was measured using Orion Diagnostica UniQ PIIINP RIA commercial kit, and HA using ELISA test kit (Corgenix UK, Cambridgeshire, UK) as per manufacturer's protocol, by a laboratory technician who was blinded to study group of participants. The intra-assay coefficient of variation (CV) for the PIIINP measurements was 4-7% across the range of concentrations measured.

5.5 Statistical analysis:

No priori power calculation was undertaken as there were no studies in the literature that used liraglutide in PCOS to base the calculation on. We are aware that interpretation of non-significant findings is challenging. Post-hoc power calculations are not recommended (384). Data were summarised by the mean and standard deviation (continuous data) or by percentages (categorical data). Data were checked for normality using Kolmogorov-Smirnov test. Between groups analysis, at baseline, was performed using the independent t-test for continuous data (or Mann-Whitney U test for non-normally distributed data).

We used intention to treat analysis in which the missing values for participants who dropped being replaced by carrying the last observation forward (351, 385). Within-group analysis (baseline vs. six months) was performed using the dependent t-test for continuous data (or Wilcoxon signed-rank test for non-normally distributed data); and we used analysis of covariance (ANCOVA) to control for change in variables when appropriate. Correlations were evaluated using Pearson's coefficient (or Spearman's coefficient for non-normally distributed data). Frequency distributions were analysed using Chi-square test. Statistical analysis was performed using the PASW statistics 19 package (SPSS Inc., Chicago, USA). A two tailed P value of <0.05 was considered statistically significant.

5.6 Results.

5.6.1 Study participants and baseline characteristics.

Fifty one women were screened, 36 recruited (19 PCOS, 17 normal controls). Fifteen women were excluded as they had; idiopathic hirsutism (five), hypothyroidism (one), BMI <30kg/m^2 (one), no trans-vaginal ultrasound (TV USS) scan was done (two), ovaries not visible on TV USS (one), oligomenorrhoea without PCOS (one), and repeatedly missing initial study visit (three), difficult venepuncture (one). Out of the 19 women with PCOS, 10 (52%) fulfilled all three Rotterdam criteria, 9 (48%) fulfilled two criteria, oligomenorrhoea and hirsutism, but no polycystic ovaries (PCO) on ultrasound.

The PCOS and control groups were well matched for age 33.9 ±6.7 vs. 33.5 ±7.1yr, weight 102.1 ±17.1 vs. 100.4 ±15.1kg, BMI 37.9 ±5.0 vs. 36.5 ±4.6 kg/m^2, and waist circumference 112.7 ±12.6 vs. 112.4 ± 9.4cm, respectively (all P >0.05). The PCOS group had higher testosterone, FAI, insulin, and HOMA-IR (Table 5.1). Other biochemical markers did not differ significantly between the two groups (Table 5.1). There was a trend but no significant difference in the presence of the metabolic syndrome; ten vs. six; 53% vs. 35% (P=0.29) for the PCOS and control groups, respectively.

	PCOS (n=19)	Controls (n=17)	P
Testosterone (nmol/L)	1.3 ±0.4	0.90 ±0.3	0.01
FAI	4.4 ±2.0	2.6 ±1.2	0.02
SHBG(nmol/L)	33.2 ±14.6	38.1 ±14.4	0.38
FPG (mmol/L)	5.1 ±0.6	4.9 ±0.4	0.22
Insulin (iu/ml)	22.0 ±9.4	16.1 ±5.6	0.03
HOMA-IR	5.1 ±2.6	3.5 ±1.3	0.03
hsCRP (mg/L)	6.2 ±8.7	5.1 ±3.4	0.63
Isoprostane (ng/ml)	0.81 ±0.53	0.58 ±0.57	0.22
Total chol (mmol/L)	5.0 ±0.8	5.1 ±1.0	0.62
LDL (mmol/L)	3.2 ±0.6	3.3 ±0.9	0.63
HDL (mmol/L)	1.2 ±0.2	1.3 ±0.2	0.25
Tri (mmol/L)	1.4 ±0.7	1.4 ±0.7	0.85
Chol/HDL	4.4 ±0.9	4.1 ±0.8	0.44

Table 5.1. Baseline biochemical and hormonal characteristics for the PCOS and control groups. FAI, free androgen index; SHBG, sex hormone binding globulin; FPG,

fasting plasma glucose; HOMA-IR, homeostatic model assessment-insulin resistance; hsCRP, high sensitivity C-reactive protein; chol, cholesterol; LDL, low density lipoprotein; HDL, high density lipoprotein; Tri, triglycerides; Chol/HDL, cholesterol to HDL ratio. Data are presented as mean ±standard deviation (SD).

NAFLD was more prevalent in the PCOS group with seven (37%) of PCOS vs. none of the controls having evidence of fatty infiltration on liver ultrasound scan (P=0.005). Aspartate aminotransferase (AST) 22.4 ±5.24 vs. 18.8 ±3.36iu/L (P=0.037), and PIIINP 4.4 ±0.8 vs. 3.5 ±0.84ug/ml (P=0.006) were also significantly higher in the PCOS group. Other clinical and biochemical markers of liver disease did not differ significantly between PCOS and controls including BARD score 2.47 ±0.91 vs. 2.41 ±0.94 (P=0.83) and F-score -1.82 ±0.98 vs. -2.39 ±0.86 (P=0.075), respectively.

5.6.2 Intervention with liraglutide.

Twenty five women, 69%, completed the study (13 PCOS, and 12 controls). Eleven women dropped out because of: nausea and vomiting (four), loss of follow up (four), frequently missing study drug (one), change in personal circumstances (one), and pregnancy (one).

At six months, weight was significantly reduced with an average of 3.0±4.2kg (P=0.01) for PCOS and 3.8 ±3.4kg (P<0.01) for controls. Similarly, insulin, FPG, HOMA-IR, hsCRP, triglycerides and urinary isoprostane significantly reduced in both groups (Table 5.2). PIIINP significantly reduced in the PCOS group only (Table 5.3). The change in PIIINP levels in the PCOS group strongly correlated with weight change (r=0.51, P=0.027), but remained significant when change in weight was adjusted for (P=0.029). Other liver markers did not differ markedly after treatment (Table 5.3).

	PCOS (n=19)			Controls (n=17)		
	Baseline	**6m**	**P**	**Baseline**	**6m**	**P**
Weight (kg)	102.1 ±17.1	99.1 ±15.9	0.01	100.4 ±15.1	96.7 ±14.4	<0.01
BMI (kg/m^2)	37.9 ±5.0	36.9 ±4.8	0.01	36.5 ±4.6	35.1 ±4.3	<0.01
Waist (cm)	112.0 ±12.6	110.9 ±12.4	0.31	112.3 ± 9.7	110.9 ±8.8	0.12
Testosterone (nmol/L)	1.3 ±0.4	1.2 ±0.5	0.78	0.90 ±0.3	0.94 ±0.2	0.63
FAI	4.4 ±2.0	4.4 ±2.7	0.86	2.6 ±1.2	2.5 ±0.8	0.78
SHBG(nmol/L)	33.2 ±14.6	30.8 ±10	0.29	38.1 ±14.4	40.3 ±12.7	0.29
FPG (mmol/L)	5.1 ±0.6	4.9 ±0.7	0.045	4.9 ±0.4	4.5 ±0.4	<0.01
Insulin (iu/ml)	22.0 ±9.4	19.5 ±9.0	0.03	16.1 ±5.6	12.8 ±4.9	0.01
HOMA-IR	5.1 ±2.6	4.3 ±2.4	0.01	3.5 ±1.3	2.6 ±1.1	<0.01
hsCRP (mg/L)	6.2 ±8.7	4.3 ±3.9	0.03	5.1 ±3.4	3.9 ±3.0	0.04
Isoprostane (ng/ml)	0.81 ±0.53	0.36 ±0.48	0.01	0.58 ±0.57	0.21 ±0.19	0.03
Total chol (mmol/L)	5.0 ±0.8	4.9 ±0.8	0.24	5.1 ±1.0	5.1 ±0.9	0.88
LDL (mmol/L)	3.2 ±0.6	3.2 ±0.6	0.38	3.3 ±0.9	3.4 ±0.8	0.51
HDL (mmol/L)	1.2 ±0.2	1.2 ±0.3	0.59	1.3 ±0.2	1.2 ±0.2	0.55
Tri (mmol/L)	1.4 ±0.7	1.1 ±0.5	<0.01	1.4 ±0.7	1.1 ±0.6	0.03
Chol/HDL	4.4 ±0.9	4.3 ±0.7	0.83	4.1 ±0.8	4.3 ±1.1	0.43
Metabolic Syndrome	53% (10)	32% (6)	0.19	35% (6)	35% (6)	1

Table 5.2 Effects of treatment with liraglutide on anthropometric, hormonal and biochemical markers in the PCOS and control groups. BMI, body mass index; FAI, free androgen index; SHBG, sex hormone binding globulin; FPG, fasting plasma glucose; HOMA-IR, homeostatic model assessment-insulin resistance; hsCRP, high sensitivity C-reactive protein. Data are presented as mean ±SD or percentages (n).

	PCOS (n=19)			Controls (n=17)		
	Baseline	**6m**	**P**	**Baseline**	**6m**	**P**
ALT (5 – 45 iu/L)	26.9 ±11.6	26.7 ±14.7	0.93	21.8 ±5.6	21.2 ±7.4	0.60
AST (8 - 28 u/L)	22.4 ±5.2	21.7 ±5.1	0.57	18.8 ±3.4	19.4 ±6.0	0.63
AST/ALT ratio	0.90 ±0.2	0.91 ±0.2	0.65	0.89 ±0.2	0.94 ±0.2	0.09
PIIINP (ug/ml)	4.4 ±0.8	3.7 ±0.9	<0.01	3.5 ±0.8	3.2 ±0.7	0.08
HA (ug/ml)	12.7 ±5.6	13.3 ±6.2	0.55	14.8 ±8.9	15.7 ±10.2	0.53

Table 5.3 Effects of treatment with liraglutide on biochemical markers of liver disease in the PCOS and control groups. ALT, alanine aminotransferase; AST, aspartate aminotransferase; PIIINP, pro-collagen type 3 amino-terminal peptide; HA, hyaluronic acid. Data are presented as mean ±SD.

5.7 Discussion.

Our data suggest that women with PCOS are at increased risk of NAFLD independent of obesity. This association between PCOS and NAFLD has also been suggested by other groups and is consistent with the increased insulin resistance in PCOS (366). NAFLD itself carries an increased risk for developing NASH and liver cirrhosis (366). In a recent validation study, Tanwar et al. (377) examined the association between biochemical markers of liver disease and liver biopsy results in 172 patients with NAFLD. A wide range of biochemical markers were measured including routine biochemistry (ALT, AST, GGT), hepatic inflammation (YKL-40, TIMP-1), apoptosis (CK-18) and liver fibrosis (HA, PIIINP, Collagen IV). The authors found PIIINP to be the single marker which differentiated between simple steatosis and NASH in patients without advanced fibrosis (377). This suggests that the significantly raised levels of PIIINP in the PCOS group in our study, compared to controls, may indicate an increased risk of developing liver fibrosis in the future in these women. Our data therefore support suggestions that evaluation for liver disease in women with PCOS may need to be undertaken at an earlier age than the general population (366).

Lifestyle changes and weight loss are the recommended treatments for NALFD (386). Six months treatment with liraglutide, in our study, resulted in a significant reduction in PIIINP in the PCOS group, resulting in values which were more

comparable with those of the controls at baseline. In contrast, liraglutide resulted in no significant reduction in PIIINP amongst the control subjects. Changes in HA levels were not significant at 6 months, which may reflect both the mild degree of liver disease in study participants and that PIIINP is a more sensitive marker for early liver fibrosis. Cuthbertson et al. (387) treated 25 obese subjects with T2DM with exenatide (n=19) or liraglutide (n=6) and found a reduction in intrahepatic fat on liver proton magnetic resonance spectroscopy after 6 months of treatment. The authors did not specify how many of their cohort had NAFLD at baseline and study participants were on other medications which may affect liver fat including metformin and dipeptidyl peptidase-IV (DPP-IV) inhibitors (387). Gangale et al. treated 70 overweight women with PCOS and NAFLD with metformin 500mg tds and found a significant reduction in AST levels and AST/ALT ratio at 6 months (379). Similarly, 8 months treatment with metformin significantly reduced levels of ALT and GGT in 66 obese women with PCOS (388). The effects of metformin on liver markers are thought to be related to improvement insulin sensitivity by reducing hepatic glucose production and increasing adenosine monophosphate-activated protein kinase-mediated oxidative glucose and lipid metabolism in hepatocytes and glucose uptake in the skeletal muscles (389). However, despite the improvement in serum ALT and AST levels reported with metformin therapy, current data suggest that metformin has no significant effect on liver histology (390); and it is not currently recommended for the treatment of NAFLD in the absence of type 2 diabetes (391). To the best of our knowledge, we are the first group to examine the use of GLP-1 analogues in obese women with PCOS at increased risk of NALFD.

As expected the use of liraglutide in our study was associated with 3 – 4% weight loss. Weight loss has been found in previous studies to improve NALFD (386) and reduce PIIINP levels in obese non-PCOS subjects (392). However, preclinical trials and animal studies suggest that GLP-1 analogues may have a role in reducing steatosis in hepatocytes (393). The effects are thought to be mediated through the GLP-1 receptor expressed on hepatocytes (394). Different mechanisms have been proposed including, increase in cyclic adenosine monophosphate (cAMP) production (395); activation of an AMP-activated protein kinase (AMPK) dependent

pathway in hepatocytes leading to an increase in fatty acid oxidation and decrease in lipogensis (396, 397); and/or an increase in hepatic insulin signaling and sensitivity with GLP-1 and subsequent improvement in hepatic glucose metabolism (394). Furthermore, liraglutide has been found to reduce fatty acid accumulation, in mice fed with high fat diet, by enhancing autophagy and reducing endoplasmic reticulum stress-related apoptosis (393). The reduction in PIIINP levels in the PCOS group in our study remained significant after adjusting for weight change. It is difficult to specify if the changes in liver fibrosis marker PIIINP noted in our study were related to liraglutide treatment *per se* or simply to the associated weight loss being more beneficial to obese women with PCOS than to controls. In addition, the long term impact of the treatment on the natural history of NALFD and its complications remains unknown. Our data suggest that liraglutide might be beneficial in NAFLD; however, larger, longer and randomised controlled trials are needed to further evaluate its effects.

Liver ultrasound scan was not repeated at 6-month of liraglutide therapy in our study as our interest was in assessing changes in liver fibrosis rather than fat content. While ultrasonography has traditionally been used to explore the liver and is able to detect changes in the liver parenchyma when there is significant fibrosis (bridging fibrosis and mainly cirrhosis) and signs of portal hypertension (enlarged spleen, collateral venous circulation, enlarged portal vein), the method is not useful for identifying patients with less advanced stages of fibrosis (398). A FibroScan, which used ultrasound in a different way, might have been helpful in assessing changes in liver fibrosis (399), however, it was not available to us.

Our study has many strengths including, the two groups were well matched for age, weight and waist circumference; we have measured sensitive and validated markers for liver fibrosis; and we have performed a prospective study using a medication which has shown beneficial effects in preclinical trials. Study weaknesses include that women with PCOS were a heterogeneous mixture of those with and without NAFLD, although this does reflect the spectrum of phenotype with this condition. Performing subgroup analysis was not possible because of the small number of participants and high rate of dropout during the study. Another

weakness is that we only examined changes in biochemical markers of liver fibrosis. Although, the inclusion of liver biopsy, the gold standard, pre/post treatment would have been more informative, all our study participants had mild degree of liver fibrosis and liver biopsy was not clinically indicated. Another limitation is that whilst it is possible that the significant drop in PIIINP in the PCOS group was related to the PCOS group having significantly higher levels of PIIINP than controls at baseline, this cannot rule out a group effect i.e. liraglutide was more effective in PCOS than controls. Lastly, this study would ideally have also included placebo treated groups for both PCOS and controls in a 4 arm study to clarify if the changes observed were related to liraglutide *per se* or to the associated weight loss. However, the results of this study would now allow a larger study to be powered appropriately to include a placebo.

5.8 Conclusions.

Obese young women with PCOS are at increased risk of NAFLD and liver fibrosis, independent of obesity. Six months treatment with liraglutide resulted in mild reduction in weight and significant improvement in insulin resistance, inflammation and the liver fibrosis marker PIIINP. Liraglutide may be a potential treatment for obese women with PCOS, obesity and NAFLD.

6. Chapter 6: The effects of treatment with liraglutide on quality of life and depression in young obese women with PCOS and controls.

6.1 Abstract.

Polycystic ovary syndrome (PCOS) is associated with conditions that may have a negative impact on quality of life (QoL) including hirsutism, oligomenorrhoea, obesity and sub-fertility. Obesity is seen in up to 78% of women with PCOS and is associated with reduced Qol. In this study our aim was to assess the effects of six months treatment with liraglutide 1.8mg od on obesity, depression and QoL in young obese women with PCOS and matched controls. We conducted an interventional case-controlled study. PCOS was diagnosed according to the Rotterdam criteria; none of the women wanted fertility. Depression was measured using the Centre for Epidemiologic Studies Depression Scale (CES-D). An average score of ≥16 on this scale suggests a high risk for depression. QoL was measured using the short version of the World Health Organization QOL questionnaire (WHOQOL-BREF) which includes four subscales (physical, psychological, social and environment). Scores are given out of a hundred and higher scores are better. Data presented as mean ±standard deviation (SD).

Thirty six women were recruited (19 PCOS, 17 controls), age 33.9 ±6.7 vs. 33.5 ±7.1yr, and weight 102.1 ±17.1 vs. 100.4 ±15.1kg, respectively (all P >0.05). The average number of periods per year was 6 vs. 12 for women with PCOS and controls, respectively. Fifteen (79%) women with PCOS complained of hirsutism. Baseline scores on the CES-D and WHOQOL-BREF did not differ between the two groups. Twenty five women, 69%, completed the study (13 PCOS, and 12 controls). Following six months treatment with liraglutide weight was reduced by 3.0 ±4.2kg, P=0.01, in the PCOS group and 3.8 ±3.4kg, P=0.001, in controls. At six months, there was no significant change in CES-D scores, compared to baseline, in either group. However, there was improvement on the WHOQOL-BREF questionnaire scores in psychological health 59.1 ±9.7 vs. 65.6 ±13.3 (P=0.02) and social health, 73.7 ±12.5 vs. 78.3 ±12.6 (P=0.06), in the PCOS group; and in physical health, 77.3 ±14.6 vs. 81.8 ±12.1 (P=0.06), and social health, 68 ±20.5 vs. 74.7 ±17.9 (P=0.08), in controls; these changes became statistically significant when both groups were combined.

In conclusions, when matched for age and obesity, PCOS was not independently associated with reduced QoL and depression, suggesting that obesity had the

greatest impact on these parameters. Six months treatment with liraglutide resulted in significant reduction in weight and improvement in QoL in young obese women.

6.2 Introduction.

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in women of reproductive age (5). While in the previous chapters I focused on the metabolic, hormonal and cardiovascular risk markers associated with the condition; in this chapter I will examine the effects of PCOS and obesity on women's psychological health and general well-being.

PCOS is associated with conditions that may have a negative impact on quality of life (QoL) including hirsutism, oligomenorrhoea, obesity and sub-fertility. While PCOS has been associated with depression and reduced QoL in several studies (400, 401), it is not yet clear which of its components has the main effect. Obesity is seen in up to 78% of women with PCOS (75) and is associated with reduced Qol (402). Despite its high prevalence, only a few studies have accounted for obesity when examining the psychological health in women with PCOS and their results are not conclusive (403, 404). In addition, the effects of weight loss on QoL in obese women with PCOS are not yet clear.

Liraglutide is a glucagon like peptide-1 analogue which causes weight loss. Although it is only currently licensed for the treatment of people with type 2 diabetes (405) it represents an attractive option for the treatment of obesity in PCOS (406). However, liraglutide is self-administered subcutaneously and individuals using injectable treatments report lower satisfaction with convenience of use compared to those using other forms of treatments e.g. oral, topical, or inhaler forms (407). The effects of treatment with liraglutide on QoL in obese women with PCOS and their satisfaction with an injectable treatment for obesity are not known.

In this study our aim was to investigate if young obese women with PCOS have increased depression and impaired QoL independent of obesity; and to examine the impact of six months treatment with liraglutide, and/or associated weight loss, on these measures. We hypothesized that PCOS was independently associated with reduced QoL and increased depression and that treatment with liraglutide would result in weight loss and improved QoL in obese women with PCOS.

6.3 Methods.

An interventional case-control study of obese women with PCOS, none of whom wanted to conceive, and age and weight matched controls. A detailed description of participants' selection criteria, study design, blood samples analysis, and statistical methods used was described in previous chapters. In brief, PCOS was diagnosed according to the Rotterdam criteria (7). Study participants were assessed at baseline and six months after treatment with liraglutide 1.8mg od. Data presented as mean ± standard deviation (SD).

6.3.1 Quality of life.

QoL was measured using the short version of the World Health Organization QoL questionnaire (WHOQOL-BREF), which includes 26 questions to assess four major domains (subscales): physical, psychological, social and environment (Table 6.1) (408). Scores are given out of a hundred and higher scores are better; a score of more than 50% indicates an acceptable to good QoL. The WHOQOL-BREF has been shown to display high internal consistency (0.92); good test-retest reliability (0.66 – 0.72); discriminant validity (discriminating sick from healthy people), and content validity (correlation with SF-36) (408). WHOQOL-BREF provides a very good holistic assessment of QoL in the general population and its validation study included a group of women with PCOS (408). However, WHOQOL-BREF may not fully address QoL in relation to PCOS specific symptoms, including body hair, infertility, and menstrual problems which are better assessed using the PCOS health-related quality of life questionnaire (PCOSQ) (409). However, none of the women in our study wanted fertility, and while we would have preferred to use both WHOQOL-BREF and PCOSQ questionnaires, the latter was not available to us and its use may not have reflected QoL in controls very well.

6.3.2 Depression.

Depression was measured using the Centre for Epidemiologic Studies Depression Scale (CES-D), which includes twenty items reflecting six major dimensions of depression: depressed mood, feelings of guilt and worthlessness, feelings of helplessness and hopelessness, psychomotor retardation, loss of appetite, and

sleep disturbance experienced in the past week (410). An average score of ≥16 on this scale suggests a high risk for depression or being in need of treatment. CES-D has been validated and it has an internal consistency ranging from 0.85 to 0.90 (410). The test-retest reliability was in the moderate range r=0.54 for tests repeated over a three to twelve month time interval (410).

6.3.3 Treatment satisfaction.

Participants' satisfaction and convenience with liraglutide was assessed at four weeks after starting study medication using the Treatment Satisfaction Questionnaire for Medication (TSQM) Version 1.4 (407). TSQM is a validated, self-reported, fourteen-item questionnaire that measures a patient's experience with medication in terms of four scales: side effects, effectiveness, convenience, and global satisfaction. Scores range from 0 to 100 with a higher score indicating greater satisfaction with treatment.

Domain	Facets incorporated within domains
1. Physical health	Activities of daily living Dependence on medicinal substances and medical aids Energy and fatigue Mobility Pain and discomfort Sleep and rest Work Capacity
2. Psychological	Bodily image and appearance Negative feelings Positive feelings Self-esteem Spirituality / Religion / Personal beliefs Thinking, learning, memory and concentration
3. Social relationships	Personal relationships Social support Sexual activity
4. Environment	Financial resources Freedom, physical safety and security Health and social care: accessibility and quality Home environment Opportunities for acquiring new information and skills Participation in and opportunities for recreation / leisure activities Physical environment (pollution / noise / traffic / climate) Transport

Table 6.1 WHOQOL-BREF domains

6.4 Results.

6.4.1 Baseline characteristic.

Thirty six women were recruited (19 PCOS, 17 controls), age 33.9 ±6.7 vs. 33.5 ±7.1yr, and weight 102.1 ±17.1 vs. 100.4 ±15.1kg, respectively (all P >0.05). The PCOS group, as expected, had higher testosterone 1.3 ±0.4 vs. 0.90 ±0.3nmol/L (P=0.01), free androgen index 4.4 ±2.0 vs. 2.6 ±1.2 (P=0.02), insulin 22.0 ±9.4 vs. 16.1 ±5.6iu/L (P=0.03), and HOMA-IR 5.1 ±2.6 vs. 3.5 ±1.3 (P=0.03), respectively.

None of the women with PCOS or controls wanted to conceive. The average number of periods per year was 6 vs. 12 for the PCOS and controls, respectively. Fifteen (79%) women with PCOS complained of hirsutism. In the PCOS group 6 participants (32%) completed secondary education, and 12 (63%) completed tertiary education, while data on education was not available on one participant (5%). In the control group 6 participants (35%) completed secondary education and 9 (53%) completed tertiary education, while data on education was not available on two participants (12%).

6.4.2 Baseline QoL and depression.

When asked how they would rate their own QoL, none of the women with PCOS or controls rated their QoL as ‘poor’ or ‘very poor’. While, two women with PCOS (11%) and four controls (24%) rated their QoL as ‘very good’ (P=0.28).

There was no significant difference between PCOS and controls on the CES-D with six (32%) vs. five (29%), P=0.80, women having scores ≥ 16 suggestive of depression, respectively. Similarly, there was no difference on the WHOQOL-BREF questionnaire, physical health 80.4 ±12.8 vs. 77.3 ±14.6 (P=0.45); psychological health 59.1 ±9.7 vs. 55.7 ±21.9 (P=0.51); social relationships 73.7 ±12.5 vs. 68 ±20.5 (P=0.32); or environment 71.5 ±12.3 vs. 73.3 ±16.9 (P=0.71) for PCOS and controls, respectively.

6.4.3 Intervention with liraglutide.

Twenty five women, 69%, completed the study (13 PCOS, and 12 controls). The 11 participants who dropped out during the study were significantly younger than

those who completed the study 30.2 ±5.2 vs. 35.2 ±6.9 years (P= 0.04), respectively, but completers and non-completers did not significantly differ in their weight, BMI, QoL or depression scores at baseline, data not presented. Reasons for drop out were: nausea and vomiting with liraglutide (four), loss of follow up (four), frequently missing study drug (one), change in personal circumstances (one), and pregnancy (one).

Following six months treatment with liraglutide weight was reduced by 3.0 ±4.2kg (P=0.01) in the PCOS group and 3.8 ±3.4kg (P=0.001) in controls. As both groups responded equally to treatment, and there was no significant difference in QoL or depression scores between the PCOS and control groups at baseline; the effects of treatment are presented for each group separately and for both groups combined.

6.4.3.1 QoL and depression.

There was no significant change after treatment in depression scores, compared to baseline, in either group or when both groups were combined (Table 6.2). There was a trend for improvement in psychological and social health scores for the PCOS group, and in physical and social health scores for controls after treatment (Table 6.2). Improvements in physical, psychological, and social health scores were significant when both groups were combined (Table 6.2).

	PCOS (n=19)			Controls (n=17)			Combined groups (n=36)		
	Baseline	**6m**	**P**	**Baseline**	**6m**	**P**	**Baseline**	**6m**	**P**
Rating own QoL as 'very good'	2 (11%)	6 (31%)	0.11	4 (24%)	4 (24%)	1	6 (17%)	10 (28%)	0.26
CES-D ≥16	6 (32%)	5 (26%)	0.72	5 (29%)	3 (18%)	0.42	11 (31%)	8 (22%)	0.42
Physical health	80.4 ±12.8	83.3 ±10.6	0.5	77.3 ±14.6	81.8 ±12.1	0.06	78.9 ±13.6	82.6 ±11.2	0.04
Psychological health	59.1 ±9.7	65.6 ±13.3	0.02	55.7 ±21.9	58.9 ±19.3	0.29	57.5 ±16.4	62.4 ±16.5	0.012
Social health	73.7 ±12.5	78.3 ±12.6	0.06	68 ±20.5	74.7 ±17.9	0.08	71 ±16.8	76.6 ±15.3	0.01
Environment	71.5 ±12.3	74.1 ±12.2	0.11	73.3 ±16.9	75.5 ±12.9	0.52	72.3 ±14.5	74.8 ±12.4	0.18

Table 6.2 Scores on the CES-D and WHOQOL-BREF questionnaires. A score of ≥16 on the Centre for Epidemiologic Studies Depression Scale (CES-D) questionnaire is suggestive of depression. There was a trend for improvement in physical, psychological, and social health domains of the WHOQOL-BREF questionnaire in both groups; these changes became statistically significant when both groups were combined. Data presented as mean ±SD or number (percentage).

6.4.3.2 Treatment satisfaction.

Thirty four participants (94%) completed the TSQM questionnaire four weeks after starting liraglutide. Data was not available in two participants who withdrew from the study prior to the assessment at four weeks because of nausea and vomiting, a common side effect when starting GLP-1 analogue therapy.

Participants in both groups reported high satisfaction with liraglutide with scores on the TSQM questionnaire's four domains (effectiveness, side-effects, convenience, and global satisfaction) of >75% (Table 6.3).

	PCOS (n=18)	Controls (n=16)	Combined (n=34)
Effectiveness	78.7 ±14.2	76.0 ±19.2	77.5 ±16.5
Side-effects	86.8 ±13.7	90.2 ±12.1	88.4 ±12.9
Convenience	87.7 ±13.9	92.3 ±9.7	89.9 ±12.2
Global satisfaction	86.1 ±14.8	80.8 ±21.8	83.6 ±18.3

Table 6.3 Scores on the TSQM questionnaire. Participants in the PCOS and control groups reported high satisfaction with liraglutide treatment with scores on the Treatment Satisfaction Questionnaire for Medication (TSQM) questionnaire of >75%.

6.5 Discussion.

Our data suggest that obesity has the bigger impact on QoL and depression in young obese women with PCOS. They also suggest that six months treatment with liraglutide results in significant reduction in weight (3 – 4%) and improvement in QoL in young obese women.

Our results are in accordance with previous data by Alvarez-Blasco et al. (403) who examined QoL in 32 obese women with PCOS and compared them to 72 controls using the Short Form 36 Health Survey (SF-36) questionnaire. The authors reported similar QoL for PCOS and controls. However, the control group in that study was significantly older (403), and older age is associated with reduced QoL (411). In

another study, Elsenbruch et al. (404) examined 50 women with PCOS and 50 age-matched controls. Although women with PCOS in that study showed significantly lower life satisfaction compared to controls, they were also significantly more overweight and many of them wanted fertility (404). Cinar et al. (412) examined 226 women with PCOS and 85 BMI matched controls using the PCOS health-related quality of life questionnaire (PCOSQ). The authors reported that menstrual and hirsutism problems were the most serious concerns followed by emotional problems in women with PCOS whereas weight and infertility were the least important. However, the average BMI of study participates was within the normal range (412). We are one of the few studies to match well for age and obesity when assessing QoL in women with PCOS. It is worth noting that none of the participants in our study wanted to conceive and that their average scores on the WHOQOL-BREF were more than 50% suggesting at least moderate – good QoL.

Only a few studies have examined the impact of weight loss on QoL and depression in women with PCOS and these studies have many limitations. Galletly et al. (413) studied 25 overweight women with PCOS and matched controls. Participants were randomized to a low-protein high-carbohydrate diet or a high-protein low-carbohydrate for 16 weeks. Depression was assessed using the Hospital Anxiety and Depression Scale and the Rosenberg Self Esteem Scale. Despite equal amounts of weight loss at the end of the study only the group who received the high-protein, low-carbohydrate diet noted improvements in their depression scores. The authors related the improvement to the type of diet received rather than to the weight loss achieved. Thomson et al. (414) examined 104 overweight or obese women with PCOS who were randomised to one of three 20-week lifestyle programs: diet only, diet and aerobic exercise, or diet and combined aerobic-resistance exercise. At the end of the study all groups achieved weight loss and improvement in depression and QoL. However, their results are limited by a high dropout rate during the study which exceeded 50% of study participants. Similarly, Marsh et al. (415) examined 96 overweight and obese women with PCOS who were randomized to low carbohydrate or conventional diet for 12 months or till achieving 7% weight loss. Study completers, ~ 50% of participants, achieved 4 – 5% weight loss and significant

improvement in QoL on the PCOSQ in both groups. Our data supports findings that weight loss improves QoL in obese women with PCOS and controls; however, we did not find significant change in depression scores.

Despite liraglutide being an injectable treatment and many study participants being resistant initially to the idea of self-injecting, participants' global satisfaction with liraglutide on the TSQM was very good. Although, the high dropout rate in our study, ~ 30%, could be in part related to the gastrointestinal side effects related to liraglutide and/or to the lack efficacy in some participants, we still believe that liraglutide, and other GLP-1 analogues, remain a potentially effective treatment for obesity.

While many studies examining QoL in women with PCOS have used the SF-36 or the PCOSQ questionnaires, we have used the WHOQOL-BREF. Although the SF-36 is still regarded by many researchers as the 'gold standard' measure for QoL, its concepts and items were proposed primarily by health researchers and clinicians rather than potential users as is the case with WHOQOL-BREF. It is therefore proposed that the WHOQOL-BREF provides a very good, more holistic cross-cultural QoL assessment (408). While the PCOSQ is a disease specific questionnaire considering 5 domains related to PCOS: emotions, body hair, weight, infertility and menstrual problems (409), these items will not accurately reflect QoL in controls.

This study has many strengths including the recruitment of two groups who were well matched for age and weight, and the use of well validated methods to assess QoL and depression. Limitations to our study include higher dropout than anticipated which may have resulted in subgroup analysis being underpowered. Combining the two groups seemed reasonable as there was no significant difference between the two groups at baseline and both groups responded equally to treatment.

6.6 Conclusions

When matched for age and obesity, PCOS was not independently associated with reduced QoL and depression, suggesting that obesity had the greatest impact on

these parameters. Six months treatment with liraglutide resulted in significant reduction in weight and improvement in QoL in young obese women.

7. Chapter 7: Summary and conclusions

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder of women of reproductive age. Obesity is strongly linked to PCOS and carries increased cardiovascular risk. Studies investigating atherothrombotic risk in women with PCOS, in particular platelet function and carotid intima-media wall thickness (cIMT), have been confounded by not adequately accounting for obesity. Liraglutide is a glucagon like peptide-1 (GLP-1) analogue that causes weight loss and may have favourable effects on atherothrombotic risk in preclinical and animal studies. However, the effect of treatment with liraglutide on platelet function and cIMT in women with PCOS and/or simple obesity is not known. The aim of this study was to investigate whether atherothrombotic risk was increased in obese women with PCOS independently of obesity, and if 6 months treatment with liraglutide would improve weight and markers of atherothrombosis in obese young women with PCOS and/or normal controls.

Our results suggest that PCOS, independent of obesity, is associated with increased levels of insulin resistance, inflammation, oxidative stress, non-alcoholic fatty liver disease (NAFLD) and the liver fibrosis marker PIIINP. However, PCOS was not independently associated with increased atherothrombotic risk markers including cIMT, platelet function, clot function/lysis, and endothelial function.

Treatment for 6 months with liraglutide, 1.8mg daily, resulted in 3 – 4% weight loss in obese women with PCOS and controls. This was associated with a significant reduction in insulin resistance, oxidative stress, and several markers of atherothrombosis including inflammation (hsCRP), serum biochemical markers of endothelial function (sP-selectin, sICAM-1, and sVCAM-1) and clot lysis. Basal platelet activation was reduced in the control group only, which may suggest an inherent defect for platelet function in PCOS. Conversely, the liver fibrosis marker PIIINP was only reduced in the PCOS group, which is probably related to the PCOS group having significantly higher levels of PIIINP than controls at baseline. No change was observed in cIMT.

We investigated the potential for liraglutide to regulate platelet function directly. Here, we demonstrated for the first time that platelets express the GLP-1 receptor (GLP-1R). Furthermore, liraglutide inhibited collagen- and thrombin-induced

aggregation in isolated platelets and the effects were at least partly mediated by the GLP-1R, although an additional GLP-1R independent pathway is also likely.

Our data highlight the need for double blind placebo controlled trials examining, the effects of liraglutide on cardiovascular outcome in people with type 2 diabetes and/or obesity, and on liver fibrosis in women with PCOS, obesity and NAFLD. In addition, further research is needed to investigate the exact mechanism of action of liraglutide in platelets.

In conclusion, cardiovascular risk in young obese women with PCOS can either be attributed to obesity or is not yet apparent at this early stage of the condition. Our data support the use of liraglutide as a weight loss medication in simple obesity and suggest a potential beneficial effect on atherothrombotic risk and markers of liver fibrosis at 6 months of treatment. GLP-1R is a novel receptor in platelets and its function and clinical effect are worth further evaluation.

In my future research, I would like to investigate the exact mechanism by which liraglutide works in platelets and to explore the effects of native GLP-1 on platelet function. I also would like to perform a placebo-controlled randomised controlled clinical trial in obese women with PCOS to examine if the changes in atherothrombotic risk and liver fibrosis markers observed with liraglutide therapy were related to liraglutide *per se* or to the associated weight loss.

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9. Appendix I

1. Isolation and preparation of human blood platelets by pH method

Buffers

- Acid citrate dextrose (ACD): 29.9mM sodium citrate, 113.8mM glucose, 72.6mM sodium chloride and 2.9mM citric acid, pH 6.4.
- Modified tyrode's: 150mM NaCl, 5mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], 0.55mM NaH2PO4, 7mM NaHCO3, 2.7mM KCl, 0.5mM MgCl2, 5.6mM glucose, pH 7.4.
- 0.3M citric acid, pH 6.5

Equipment

- Butterfly-21 Venisystems (Abbot Laboratories)
- Falcon Tubes (Falcon, Becton Dickinson)
- Centrifuge (Universal 320, Hettich)

2. Determination of platelet concentration

Buffers

- Ammonium oxalate: Ammonium oxalate (1% w/v) in dH_2O.

Equipment

- Improved Neubauer cell counter.
- Inverted light microscope.

3. Measurement of platelet aggregation

Buffers

- Modified Tyrode's buffer: NaCl (137mM), KCl (2.7mM), MgCl2 (1mM), Glucose (5.6mM), NaH2PO4 (3.3mM), HEPES (20mM), pH 7.4

Equipment

- Aggregation Module-Dual Channel (Payton)
- Aggregation cuvettes

4. Measurement of protein concentration:

Buffers

- Lysis buffer containing phosphatase and protease inhibitors: NaCl (150mM), Tris base (10mM), EDTA (1mM), EGTA (10mM).

Assay kit

- DC protein assay kit (Bio-Rad)

Equipment

- Costar 96-well cell culture plate (Corning Incorporated)
- Multiplate reader with 750nm filter

5. Western blotting

Sample preparation:

Buffers:

- Laemmli sample buffer (2x): Tris base (50mM), SDS (4% w/v), Glycerol (20% v/v), bromophenol blue (trace), 2-mercaptoethanol (5% v/v) pH 6.8.

Equipment:

- Aggregation Module-Dual Channel (Payton)
- Aggregation cuvettes

Protein separation by electrophoresis:

Buffers:

- Buffer I: Tris base (0.5M), SDS (0.4% w/v), pH 8.8.
- Buffer II: Tris base (1.5M), SDS (0.4% w/v), pH 6.8.
- Ammonium persulfate (APS): APS (10% w/v) in dH 2O
- Running buffer: Tris base (25mM), Glycine (192mM), SDS (0.1% w/v)

Equipment:

- Miniprotean 3 Cell (Bio-Rad, UK)

- Gradient mixer (Bio-Rad, UK)
- Peristaltic pump
- Butterfly-21 Venisystems (Abbot Laboratories)
- Plastic tubing
- Biotin-protein ladder (Cell Signaling Tech, UK)

Gradient gel compositions for 1.5mm casting plates:

Compound	**3% stacking gel**	**10% resolving gel**	**18% resolving gel**
dH_2O	4.87ml	1.418ml	0,708ml
Acrylamide 30%	0.75ml	1.182ml	1.961ml
Buffer I	---	0.886ml	0.886ml
Buffer II	1.87ml	---	---
APS 10%	75µl	18µl	18µl
TEMED	10µl	2µl	2µl

Immunoblotting:

Buffers:

- Transfer buffer: Tris base (25mM), Glycine (192mM), methanol (20% v/v).
- Tris buffered saline containing Tween (0.1%): NaCI (100mM), Tris base (10mM), Tween 20 (0.1% v/v), pH 7.4.
- RestoreTM Plus Wester Blot Stripping buffer: from Thermo Scientific.
- ECL 1: Luminol (250mM), p-coumaric acid (90mM), Tris base (100mM, pH 8.5), in 100ml using dH_20.
- ECL 2: Tris base (100mM, pH 8.5), 64ul of H2 O 2 (30%), in 100ml using dH_2O.
- ECL 1 and ECL 2 were mixed fresh at a ratio of 1:1 before use.
- Developing solution: diluted 1:5 prior to use in dH_2O
- Fixing solution: diluted 1:5 prior to use in dH_2O

Equipment:

- Hybond-P PVDF membrane (Amersham Pharmacia Biotech)
- Mini Trans-Blot elctroph. transfer cell (Bio-Rad, UK)

- Exposure cassette (Sigma Ltd. Poole, UK)
- Hyper film (Amersham Biosciences, UK)
- Microplate shaker

6. Flow cytometry:

Buffers:

- Sodium Citrate: Trisodium citrate (16.04g), dH_2O (500ml), PH to 7.4. Ratio when mixed with whole blood 1:8 (vol:vol).
- Modified Tyrodes: 150mM NaCl, 5mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], 0.55mM NaH2PO4, 7mM NaHCO3, 2.7mM KCl, 0.5mM MgCl2, 5.6mM glucose, pH 7.4.
- Formaldehyde (0.2%): Sodium chloride (0.9g), dH2O (100ml), formaldehyde 37% (540.5ul).
- Lysis buffer: BD FACS Lysing Solution 10X Concentrate (BD Biosciences). Lysis buffer was diluted (1:9 vol:vol) by dH_2O before usage.

Equipment:

- Flow cytometer (FACSAria II, BD Biosciences)

7. Phospho-flow:

Equipment:

- Flow cytometer (FACSAria II, BD Biosciences)

8. Culture of Human Umbilical Vein Endothelial cells (HUVECs):

Buffers:

- Lysis buffer: Tris base (50mM), SDS (4% w/v), Glycerol (20% v/v), pH 6.8.

Material:

- Medium 200 (GIBCO DMEM, Life technologies, USA) supplemented with Low Serum Growth Supplement (LSGS) (Invitrogen)
- Trypsin/EDTA solution (Invitrogen)

- Trypsin Neutralizer solution (Invitrogen)
- Attachment factor (Invitrogen)
- Trypan blue solution
- T-75 flask: 75 cm^2 tissue culture flasks.
- 6-well culture plate
- Sterile Pipettes
- Centrifuge
- Flow culture hood
- Humidified cell culture incubator
- Haemocytometer
- 70% ethanol for cleaning
- 15ml falcon tube

10. Appendix II

1. List of inhibitors / activators

Compound	Supplier
EGTA	Sigma Aldrich
Exendin 9-39	Sigma Aldrich
Liraglutide	Novo Nordisk
PGE1	Sigma Aldrich
PGI2	Cayman Chemicals
Collagen	Nycomed (Alexix Sheild UK)
Thrombin	Sigma Aldrich
ADP	Sigma Aldrich

2. Antibody List:

Antibody	Company
Anti-biotin-protein ladder	Cell signalling
Anti-β-Tubulin (mouse monoclonal)	Millipore
Anti GLP-1R (H-55) (rabbit polyclonal antibody)	Santa Cruz
Anti-mouse IgG-HRP	Amersham
Anti-phospho-VASP (Ser 157) (rabbit polyclonal)	Cell Signaling
Anti-phospho-PKA substrate (RRXS/T) (rabbit monoclonal)	Cell Signaling
FITC Anti-human CD42a (mouse IgG_{1K} monoclonal antibody)	BD Biosciences
FITC Anti-human CD42b (mouse $IgG_{1\kappa}$ monoclonal antibody)	BD Biosciences
FITC Anti-human fibrinogen (rabbit polyclonal antibody)	DacoCytomation
FITC Isotype control (mouse $IgG_{1\kappa}$ monoclonal antibody)	BD Biosciences
Goat Anti-rabbit IgG-HRP	Amersham
PE Anti-human CD14 (mouse $IgG_{2a\kappa}$ monoclonal antibody)	BD Biosciences
PE Anti-human CD45 (mouse $IgG_{1\kappa}$ monoclonal antibody)	BD Biosciences
PE Anti-human CD62P (mouse $IgG_{1\kappa}$ monoclonal antibody)	BD Biosciences
PE Anti-human GLP-1R (mouse IgG_{2B} monoclonal antibody)	R&D systems
PE Isotype control (mouse $IgG_{1\kappa}$ monoclonal antibody)	BD Biosciences

11. WHOQOL-BREF

12. Centre for Epidemiologic Studies Depression Scale

Center for Epidemiologic Studies Depression Scale (CES-D)

Below is a list of some of the ways you may have felt or behaved. Please indicate how often you have felt this way during the **past week**: *(circle **one** number on each line)*

During the past week...	**Rarely or none of the time** (less than 1 day)	**Some or a little of the time** (1-2 days)	**Occasionally or a moderate amount of time** (3-4 days)	**All of the time** (5-7days)
1. I was bothered by things that usually don't bother me	0	1	2	3
2. I did not feel like eating; my appetite was poor	0	1	2	3
3. I felt that I could not shake off the blues even with help from my family	0	1	2	3
4. I felt that I was just as good as other people	0	1	2	3
5. I had trouble keeping my mind on what I was doing	0	1	2	3
6. I felt depressed	0	1	2	3
7. I felt that everything I did was an effort	0	1	2	3
8. I felt hopeful about the future	0	1	2	3
9. I thought my life had been a failure	0	1	2	3
10. I felt fearful	0	1	2	3
11. My sleep was restless	0	1	2	3
12. I was happy	0	1	2	3
13. I talked less than usual	0	1	2	3
14. I felt lonely	0	1	2	3
15. People were unfriendly	0	1	2	3

During the past week...	Rarely or none of the time (less than 1 day)	Some or a little of the time (1-2 days)	Occasionally or a moderate amount of time (3-4 days)	All of the time (5-7days)
16. I enjoyed life	0	1	2	3
17. I had crying spells	0	1	2	3
18. I felt sad	0	1	2	3
19. I felt that people disliked me	0	1	2	3
20. I could not "get going"	0	1	2	3

Scoring

Item Weights	Rarely or none of the time (less than 1 day)	Some of a little of the time (1-2 days)	Occasionally or a moderate amount of the time (3-4 days)	All of the time (5-7 days)
Items 4, 8, 12, & 16	3	2	1	0
All other items:	0	1	2	3

Score is the sum of the 20 item weights. If more than 4 items are missing, do not score the scale. A score of 16 or greater is considered depressed.

Characteristics

Tested on 175 subjects.

No. of items	Observed Range	Mean	Standard Deviation	Internal Consistency Reliability	Test-Retest Reliability
20	1-53	16.2	10.9	.91	NA

Source of Psychometric Data

Stanford Arthritis Self-Management Study, 1996. Unpublished.

Comments

We are no longer using the CES-D in multiethnic studies because we have found that the norms for various ethnic groups differ. This scale is available in Spanish.

References

Radloff LS, The CES-D scale: A self-report depression scale for research in the general population. *Applied Psychological Measurement*, 1, 1977, pp.385-401.

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13. Treatment Satisfaction Questionnaire for Medicine (TSQM)