Shilpa Lingaiah

MARKERS ASSESSING BONE AND METABOLIC HEALTH IN POLYCYSTIC OVARY SYNDROME
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Academic dissertation to be presented with the assent of the Doctoral Training Committee of Health and Biosciences of the University of Oulu for public defence in Auditorium 4 of Oulu University Hospital, on 16 April 2021, at 12 noon

UNIVERSITY OF OULU, OULU 2021
Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in reproductive-aged women. The main features of the syndrome include menstrual irregularities, hyperandrogenism, polycystic ovaries, chronic inflammation, and insulin resistance, predisposing these women to reproductive, metabolic, and cardiovascular health implications across the life span. Adipose tissue dysfunction, via the secretion of adipocytokines and altered intestinal permeability have been thought to influence the pathogenesis of the syndrome. Moreover, women with PCOS display changes in several hormonal systems, which can affect bone metabolism and bone mass.

The main objectives of the study were to evaluate the association between PCOS and bone metabolism, and to study the effect of metformin, a common drug used in the management of PCOS, on bone health in women with PCOS. Further, adipokines and intestinal permeability markers were studied to evaluate their association in women with PCOS. The study populations were a Nordic population including 298 women with PCOS and 194 controls, and a population derived from the Northern Finland Birth Cohort 1966 consisting of 104 women with PCOS and 203 controls.

Decreased levels of bone formation markers and unchanged levels of bone resorption marker were observed in women with PCOS under the age of 30 compared with age-matched controls. Furthermore, metformin treatment in premenopausal women with PCOS for three months was associated with reduced bone turnover as assessed by decreases in bone formation and resorption markers. These findings suggest that bone formation is decreased in women with PCOS under the age of 30. However, treatment with metformin was associated with reduced bone turnover and slower bone remodeling, possibly preventing bone loss. Even though increased levels of retinol-binding protein 4 (RBP4), an adipokine influencing systemic insulin sensitivity, were seen in women with PCOS under the age of 30, the impact of RBP4 on the metabolic derangements in PCOS could not be determined. RBP4 does not seem to be a marker of insulin resistance or other metabolic derangements in women with PCOS. Serum levels of markers of intestinal permeability (IP) and dysbiosis did not differ between body mass index-matched women with PCOS and controls at late reproductive age, suggesting that IP and dysbiosis do not influence the metabolic derangements in women with PCOS.

**Keywords:** adipokines, bone formation, bone resorption, bone turnover markers, carboxy-terminal cross-linking telopeptide of type I collagen, fatty acid-binding protein 2, indican, intestinal permeability, metformin, osteocalcin, polycystic ovary syndrome, procollagen type I amino-terminal propeptide, retinol-binding protein 4, zonulin
Lingaiah, Shilpa, Luun ja metabolisen terveyden merkkiaineet monirakkulaisessa munasarjaoireyhtymässä.

Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta; Medical Research Center Oulu; Oulun yliopistollinen sairaala

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Tiivistelmä


Rasvakudoksen toimintahäiriöiden, jotka välittyvät adiposytokcin ja muuttuneen suoliston läpäisevyyden kautta, on uskottu liittyvän oireyhtymän patogeneesiin. Lisäksi PCOS-naisilla esiintyy häiriöitä useissa hormonaalisissa järjestelmissä, jotka voivat vaikuttaa luun aineenvaihduntaan ja luumassaan.


Asiakset: adiposytokin, regulaani, luun resorptio, luun vaihtuvuusmerkkiaineet, luumannouduus, metformiini, monirakkulaiset munasarjar, osteokalsiini, rasvahappoja sitova proteiini 2, retinolia sitova proteiini 4, suoliston läpäisevyys, tyypin I kollageenin karboksiterminaalinen telopeptidi, tyypin I prokollageenin aminoterminaalinen propeptidi, zonuliini
To my family
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Oulu, January 2021

Shilpa Lingaiah
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>25OHD</td>
<td>25-hydroxyvitamin D</td>
</tr>
<tr>
<td>ADA</td>
<td>American Diabetes Association</td>
</tr>
<tr>
<td>AE-PCOS</td>
<td>androgen excess and polycystic ovary syndrome</td>
</tr>
<tr>
<td>ALP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>AMPK</td>
<td>adenosine monophosphate-activated protein kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ASRM</td>
<td>American Society of Reproductive Medicine</td>
</tr>
<tr>
<td>BALP</td>
<td>bone alkaline phosphatase</td>
</tr>
<tr>
<td>BMD</td>
<td>bone mineral density</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>BTMs</td>
<td>bone turnover markers</td>
</tr>
<tr>
<td>cFT</td>
<td>calculated free testosterone</td>
</tr>
<tr>
<td>CLIA</td>
<td>chemiluminescence immunoassay</td>
</tr>
<tr>
<td>CTX</td>
<td>carboxy-terminal cross-linking telopeptide of type I collagen</td>
</tr>
<tr>
<td>CV</td>
<td>coefficient of variation</td>
</tr>
<tr>
<td>DHEAS</td>
<td>dehydroepiandrosterone sulfate</td>
</tr>
<tr>
<td>DPD</td>
<td>deoxypyridinoline</td>
</tr>
<tr>
<td>DXA</td>
<td>dual-energy X-ray absorptiometry</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ESHRE</td>
<td>European Society of Human Reproduction and Embryology</td>
</tr>
<tr>
<td>FABP2</td>
<td>fatty acid-binding protein 2</td>
</tr>
<tr>
<td>FAI</td>
<td>free androgen index</td>
</tr>
<tr>
<td>FG</td>
<td>Ferriman-Gallwey</td>
</tr>
<tr>
<td>GH</td>
<td>growth hormone</td>
</tr>
<tr>
<td>GLUT4</td>
<td>glucose transporter type 4 protein</td>
</tr>
<tr>
<td>GnRH</td>
<td>gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>HA</td>
<td>hyperandrogenism</td>
</tr>
<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>homeostasis model assessment of insulin resistance</td>
</tr>
<tr>
<td>hs-CRP</td>
<td>high-sensitivity C-reactive protein</td>
</tr>
<tr>
<td>ICTP/CTX-MMP</td>
<td>carboxy-terminal telopeptide of type I collagen generated by metalloproteinases</td>
</tr>
</tbody>
</table>
IFCC  International Federation of Clinical Chemistry and Laboratory Medicine
IFG  impaired fasting glucose
IGF  insulin-like growth factor
IGF-1  insulin-like growth factor-1
IGT  impaired glucose tolerance
IOF  International Osteoporosis Foundation
IP  intestinal permeability
IR  insulin resistance
IRS-1  insulin receptor substrate-1
ISI  insulin sensitivity index
LDL  low-density lipoprotein
LH  luteinizing hormone
MAPK  mitogen-activated protein kinase
MetS  metabolic syndrome
MHz  megahertz
NFBC  Northern Finland Birth Cohort
NGT  normal glucose tolerance
NIH  National Institutes of Health
NTX  amino-terminal cross-linking telopeptide of type I collagen
OA  oligo-anovulation
OC  osteocalcin
OGTT  oral glucose tolerance test
PCOM  polycystic ovarian morphology
PCOS  polycystic ovary syndrome
PEPCK  phosphoenolpyruvate carboxykinase
PI3K  phosphatidylinositol 3-kinase
PYD  pyridinoline
PICP  procollagen type I carboxy-terminal propeptide
PINP  procollagen type I amino-terminal propeptide
QCT  quantitative computed tomography
RBP4  retinol-binding protein 4
SHBG  sex hormone-binding globulin
T  testosterone
T2DM  type 2 diabetes mellitus
TJ  tight junction
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<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>TRAP</td>
<td>tartrate-resistant acid phosphatase</td>
</tr>
<tr>
<td>WHR</td>
<td>waist-hip ratio</td>
</tr>
</tbody>
</table>
List of original publications

This thesis is based on the following publications, which are referred to throughout the text by their Roman numerals:


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1 Introduction

Polycystic ovary syndrome (PCOS) is a heterogeneous disorder that includes reproductive, metabolic, and cardiovascular components with health implications across the life span. The prevalence of PCOS ranges from 5% to 20% depending on the diagnostic criteria used, and it is the most common endocrine disorder in reproductive-aged women (Asunción et al., 2000; Azziz et al., 2016; Azziz et al., 2004b; Yildiz, Bozdag, Yapici, Esinler, & Yarali, 2012). According to Rotterdam criteria, PCOS is defined as the presence of two of the following three features: oligo and/or anovulation, biochemical or clinical hyperandrogenism and polycystic ovarian morphology in ultrasonography (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004). The precise etiology of PCOS is unknown but it is considered to be a multifactorial disorder involving genetic, metabolic and environmental factors (Escobar-Morreale, 2018).

Adipose tissue dysfunction is thought to play a significant role in the metabolic abnormalities in women with PCOS (Villa & Pratley, 2011). Adipose tissue acts as an endocrine organ secreting various adipokines, including leptin, adiponectin and retinol-binding protein 4 (RBP4) (Ouchi, Parker, Lugus, & Walsh, 2011). The possible role of adipokines in the pathogenesis of PCOS is unclear. Although studies have linked RBP4 with systemic insulin sensitivity and glucose homeostasis (Graham et al., 2006; Wolf, 2007; Q. Yang et al., 2005), the association of RBP4 with metabolic derangements in women with PCOS is controversial. Furthermore, the role of gut barrier dysfunction has been considered in the pathogenesis of PCOS, as increased intestinal permeability is thought to provoke a chronic inflammatory response leading to increased ovarian androgen synthesis (Tremellen & Pearce, 2012).

Bone mineral density (BMD), a predictive factor for osteoporosis, is dependent on accumulated bone mass and rate of bone loss (Lane, 2006). Peak bone mass is usually reached from late adolescence to early thirties and is influenced by mechanical loading and reproductive hormones (Chew & Clarke, 2018; Recker et al., 1992; Weaver et al., 2016). Women with PCOS display changes in several hormonal systems, which can affect bone metabolism and bone mass (Krishnan & Muthusami, 2017). The primary focus of studies on PCOS has been on the metabolic consequences, while conclusive data on BMD and bone health are still lacking. Studies have reported conflicting results as regards BMD and fracture risk in women with PCOS (Berberoglu, Aktas, Fidan, Yazici, & Aral, 2015; Karadağ, Yoldemir, & Gogas Yavuz, 2017; Kassanos et al., 2010; Katulski et al., 2014; McBreairty et al., 2018; Rubin, Glintborg, Nybo, Andersen, & Abrahamsen, 2016; H. Y. Yang et al., 2018). Clarification of the role of PCOS as regards
the risk of developing bone frailty is vital, considering the prevalence of PCOS and the burden of osteoporosis.

Metformin is one of the most widely used drugs for the alleviation of metabolic disturbances in PCOS. Cellular and animal studies have reported that metformin has a direct osteogenic effect and a bone loss inhibiting effect (Gilbert & Pratley, 2015). However, data regarding its effect on bone metabolism in women with PCOS is lacking. As a commonly prescribed drug, it would be interesting to know if metformin modulates bone metabolism, thus influencing BMD in women with PCOS.

Bone mass has traditionally been evaluated by densitometric techniques, which are considered to be static measures. On the other hand, bone turnover markers (BTMs) assess the dynamics of metabolic imbalances of skeletal pathology (Seibel, 2005). BTMs reflect underlying changes in bone mass and bone histomorphometry parameters and are thus predictive of whole-body bone turnover. Furthermore, BTMs are sensitive enough to measure acute changes in bone turnover, providing a representative view of overall bone loss compared with that obtained by measurement of BMD at specific skeletal sites (Delmas, Eastell, Garnero, Seibel, & Stepan, 2000). There is a moderate association between baseline BTM levels and subsequent changes in BMD (Ivaska et al., 2008). These findings imply that BTMs can be used as surrogate measures to evaluate bone health.
2 Review of literature

2.1 Polycystic ovary syndrome (PCOS)

2.1.1 Diagnostic criteria and prevalence

In 1935, Irving F. Stein and Michael L. Leventhal reported a series of women who presented with amenorrhea, hirsutism, obesity, and a characteristic polycystic appearance of the ovaries seen in transabdominal pneumoperitoneum and laparotomy with wedge resection of the ovaries (Stein & Leventhal, 1935). This was one of the initial descriptions of a complex phenotype, known today as polycystic ovary syndrome. Since then, several diagnostic criteria have been developed to define the syndrome. In 1990, an expert conference sponsored by the National Institutes of Health (NIH) framed the first international diagnostic criteria for PCOS. The syndrome was defined by clinical and/or biochemical hyperandrogenism (HA) and chronic anovulation after the exclusion of other endocrine disorders such as non-classic adrenal hyperplasia, thyroid dysfunction, hyperprolactinemia and androgen-secreting neoplasms (Zawadzki & Dunaif, 1992). However, it was recognized that some women with PCOS displayed ovarian dysfunction without androgen excess, thus necessitating broader diagnostic criteria.

The diagnostic criteria for PCOS were updated in 2003 in Rotterdam by the European Society for Human Reproduction and Embryology (ESHRE) and the American Society for Reproductive Medicine (ASRM) expert conference. Ultrasonographic characteristics of polycystic ovarian morphology (PCOM) were added to the NIH criteria. The revised criteria required at least two of the following three features for the diagnosis: 1) oligo- or anovulation (OA), 2) clinical and/or biochemical signs of HA, and 3) polycystic ovaries after exclusion of other etiologies (congenital adrenal hyperplasia, androgen-secreting tumors, Cushing’s syndrome) (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004). The introduction of Rotterdam criteria led to a significant increase in the number of women being diagnosed with PCOS. Furthermore, it expanded the heterogeneity of PCOS phenotypes compared with the NIH criteria (Broekmans et al., 2006).

In 2006, a third recommendation for the diagnosis for PCOS was published by the Androgen Excess and PCOS (AE-PCOS) Society, which emphasized the importance of HA in line with NIH guidelines and ascertained that PCOS should
be primarily considered as a syndrome of androgen excess (Azziz et al., 2006). The AE-PCOS society criteria were defined as 1) HA (hirsutism and/or hyperandrogenemia), 2) ovarian dysfunction (OA and/or PCOM) and 3) exclusion of other causes of androgen excess or related disorders such as 21-hydroxylase-deficient non-classic adrenal hyperplasia, androgen-secreting neoplasms, androgenic/anabolic drug use or abuse, Cushing’s syndrome, the syndromes of severe insulin resistance, thyroid dysfunction, and hyperprolactinemia. The three different diagnostic criteria for the diagnosis of PCOS are described in Table 1.

Table 1. Diagnostic criteria for defining PCOS.

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Features</th>
<th>Required</th>
</tr>
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<tbody>
<tr>
<td>NIH 1990</td>
<td>HA, OA</td>
<td>Two of two</td>
</tr>
<tr>
<td>Rotterdam 2003</td>
<td>HA, OA, PCOM†</td>
<td>Two of three</td>
</tr>
<tr>
<td>AE-PCOS Society 2006</td>
<td>HA, Ovarian dysfunction (OA and/or PCOM)</td>
<td>Two of two</td>
</tr>
</tbody>
</table>


†According to the international evidence-based guidelines, the revised PCOM criteria are ≥ 20 follicles per ovary instead of ≥ 12 follicles per ovary (Teede et al., 2018).

In 2012, the Evidence-based Methodology Workshop on PCOS by the NIH consensus panel recommended use of the broader 2003 Rotterdam criteria while specifically identifying the PCOS phenotype (National Institutes of Health, 2012). As previously proposed by the AE Society (Azziz et al., 2006), the NIH consensus panel recommended use of the following phenotype classification: phenotype A: HA, OA and PCOM, phenotype B: HA and OA, phenotype C: HA and PCOM, and phenotype D: OA and PCOM (National Institutes of Health, 2012). The PCOS phenotypes and their associations with the different diagnostic criteria are given in Table 2.

International evidence-based guideline for the assessment and management of PCOS, published in 2018, endorsed the Rotterdam PCOS diagnostic criteria in adults (two of the following: OA, HA or PCOM), after exclusion of related disorders. The guidelines emphasized that when both OA and HA were present, ultrasonographic assessment of the ovaries was not necessary for diagnosis, and different diagnostic criteria for PCOM should be used when using older and newer ultrasonographic techniques (Teede et al., 2018).
Table 2. Classification of phenotypes of PCOS.

<table>
<thead>
<tr>
<th>Features/ Criteria</th>
<th>Phenotype A</th>
<th>Phenotype B</th>
<th>Phenotype C</th>
<th>Phenotype D</th>
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<tbody>
<tr>
<td>HA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>OA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PCOM</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NIH 1990</td>
<td>×</td>
<td>×</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>Rotterdam 2003</td>
<td>×</td>
<td>×</td>
<td>×</td>
<td>×</td>
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<tr>
<td>AE-PCOS Society</td>
<td>×</td>
<td>×</td>
<td>×</td>
<td>+</td>
</tr>
</tbody>
</table>


Prevalence of PCOS

The prevalence of PCOS varies significantly according to the diagnostic criteria used, being 4.6–10.7% based on NIH criteria, 9.5–19.9% according to the Rotterdam criteria and 8–15.3% based on AE-PCOS Society criteria (Asunción et al., 2000; Azziz et al., 2004b; Bozdag, Mumusoglu, Zengin, Karabulut, & Yildiz, 2016; Knochenhauer et al., 1998; Lauritsen et al., 2014; W. A. March et al., 2010; Yildiz et al., 2012). The variations in the reported prevalence rates involving the same subset of diagnostic criteria may, in part, be explained by differences in study populations, including ethnicity, limitations within the sampling procedures, and the application of varying methods to evaluate key PCOS features.

2.1.2 Clinical characteristics

Menstrual irregularities and ovulatory dysfunction

Chronic anovulation is usually manifested as oligomenorrhea (menstrual cycle length of > 35 days or < 8 cycles in a year) or amenorrhea (absence of menstruation for > 3 months without pregnancy) (Norman, Dewailly, Legro, & Hickey, 2007). Oligo-amenorrhea is a common feature of PCOS, seen in approximately 75–85% of women diagnosed with the syndrome (Azziz et al., 2004b). Of note, the prevalence of menstrual dysfunction decreases with age as the women approach menopause (Elting, Korsen, Rekers-Mombarg, & Schoemaker, 2000). Anovulation in PCOS is characterized by the arrest of antral-follicle growth, thus impairing the emergence of a dominant follicle. Follicular arrest is associated with hypersecretion
of luteinizing hormone (LH) and insulin, in addition to a hyperandrogenic environment (Franks, Stark, & Hardy, 2008).

**Hyperandrogenism**

Hyperandrogenism (HA) in PCOS can be assessed by clinical features (clinical HA) or biochemical indices (biochemical HA).

Clinical manifestations of HA include hirsutism (male-type terminal hair growth), acne and androgenic alopecia (male-pattern hair loss). Hirsutism is present in approximately 80% of women with HA and is considered the most reliable clinical indicator of HA. Acne and alopecia are nonspecific features. Hirsutism is an important feature of PCOS, affecting about 65−75% of women with the condition (Azziz et al., 2009; Diamanti-Kandarakis & Panidis, 2007; Hahn et al., 2005; Legro et al., 2006). Hirsutism is clinically evaluated by using the Ferriman–Gallwey (FG) scoring method, which scores (from 0−4) hair growth in eleven body areas (upper lip, chin, chest, upper and lower back, upper arm, forearm, upper and lower abdomen, thighs and lower legs) (Ferriman & Gallwey, 1961). At present, the most commonly used method to score hirsutism is a modification of the original FG method, which scores nine body areas (Yildiz, Bolour, Woods, Moore, & Azziz, 2010) (Figure 1). An FG score of > 7 defines hirsutism.

Biochemical HA, also known as hyperandrogenemia, refers to excess circulating androgen levels. Biochemical HA is assessed by measuring the levels of serum testosterone (T) or calculated indices of androgenicity, such as the free androgen index (FAI) and calculated free testosterone (cFT). The FAI is calculated from serum concentrations of T and sex hormone-binding globulin (SHBG). cFT takes into account plasma albumin concentrations along with SHBG and total T and it correlates well with free T concentrations measured by equilibrium dialysis, which is considered to be the most sensitive method of analysis of T (Miller et al., 2004; Vermeulen, Verdronck, & Kaufman, 1999). Liquid chromatography-mass spectrometry and extraction/chromatography immunoassays are the preferred assays for T measurement. The Rotterdam consensus recommended the use of cFT or the FAI for assessing biochemical HA in PCOS (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004). Along with these, international evidence-based guideline also recommends calculated bioavailable T (Teede et al., 2018).
Polycystic ovarian morphology

The criteria for defining polycystic ovarian morphology (PCOM) have been modified over the years as a result of technical advancements in ultrasonography. PCOM was initially described as the presence at least ten follicles of 2–8 mm diameter in one plane and/or increased ovarian volume (> 10 mL) in at least one ovary as visualized in transabdominal ultrasonography (Adams, Polson, & Franks, 1986). The Rotterdam consensus defined PCOM as the presence of 12 or more follicles measuring 2–9 mm in diameter and/or increased ovarian volume (>10 mL) in at least one ovary (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004). The AE-PCOS Society task force recommended using a follicle count of ≥ 25 per ovary for defining PCOM when using newer technology (i.e., transducer frequency ≥ 8 MHz) (Dewailly et al., 2014). International evidence-based guideline for the assessment and management of PCOS revised the threshold for PCOM as a follicle number per ovary of ≥ 20 and/or an ovarian
volume \( \geq 10 \text{ mL} \) in either ovary when using newer transducers (Teede et al., 2018). Polycystic ovaries, detected by transvaginal ultrasonography, may be found in about 75% of women with a clinical diagnosis of PCOS (Azziz et al., 2006). Furthermore, PCOM in ultrasonography has been observed in 14–32% of regularly menstruating healthy women (Clayton et al., 1992; Dewailly et al., 2014; Johnstone et al., 2010; Koivunen et al., 1999).

In PCOS, the ovarian anatomy is abnormal, as polycystic ovaries display a sixfold greater density of preantral follicles, including those at primordial and primary stages, compared with normal ovaries (Webber et al., 2003). Antral follicle growth is arrested when the follicle is 5–8 mm in diameter, resulting in the accumulation of multiple preantral and antral follicles in the ovarian cortex, thus generating the typical appearance of polycystic ovaries. Premature arrest of follicles is associated with hypersecretion of LH, insulin and anti-Müllerian hormone (AMH), together with a hyperandrogenic environment (Franks et al., 2008).

**Other features of PCOS**

**Obesity**

Obesity, a growing global health problem, is a common feature seen in women with PCOS that profoundly affects the clinical and biochemical presentation of the condition (Barber, McCarthy, Wass, & Franks, 2006). The reported prevalence of obesity among women with PCOS varies widely between populations, being 30–88% and this most likely reflects geographical and racial variability (Azziz et al., 2004a; Carmina, Rosato, Janni, Rizzo, & Longo, 2006; Legro, 2000). Obesity exacerbates the metabolic and reproductive abnormalities of the syndrome, especially cardiovascular risk factors such as glucose intolerance and dyslipidemia (Legro, 2012). Women with PCOS are more likely to present with an abdominal or visceral pattern of fat distribution, which is associated with a more adverse metabolic profile including greater degrees of insulin resistance (IR) and glucose intolerance compared with subcutaneous adipose tissue (Escobar-Morreale & San Millán, 2007; C. S. Fox et al., 2007; Kirchengast & Huber, 2001; J. Lord, Thomas, Fox, Acharya, & Wilkin, 2006). Thus, adipose tissue distribution may be more pertinent to metabolic health, perhaps even more than body mass index (BMI) *per se*. Waist circumference and the waist-hip ratio (WHR) are the commonly used
measures to evaluate abdominal adiposity. Of note, waist circumference is considered a better predictor of abdominal adiposity (Ping et al., 2018; Ross et al., 2020).

**Insulin resistance**

Insulin resistance (IR) refers to decreased sensitivity of target tissues to the metabolic actions of insulin, most commonly decreased insulin-mediated glucose uptake. Consequently, the pancreas increases insulin production to maintain normal glucose homeostasis leading to compensatory hyperinsulinemia (Legro, Castracane, & Kauffman, 2004). IR is a common finding seen in 50−70% of women with PCOS (Ovalle & Azziz, 2002). It is due to an intrinsic defect in the insulin signaling pathway in the skeletal muscle and adipocytes, primary target tissues of insulin action (Diamanti-Kandarakis & Dunai, 2012). Furthermore, there is a deleterious synergistic effect of obesity, particularly visceral obesity, and PCOS, which further impairs glucose tolerance and promotes IR (Escobar-Morreale, 2018). Of note, IR is also seen in lean women with PCOS (Hansen et al., 2019; Morciano et al., 2014; Stepto et al., 2013). Women with PCOS are at an increased risk of impaired glucose tolerance (IGT) and type 2 diabetes mellitus (T2DM) (Ehrmann, 2005).

The gold standard technique for assessing IR is the euglycemic-hyperinsulinemic clamp (DeFronzo, Tobin, & Andres, 1979), which is time-consuming and expensive. Hence, insulin indices derived from fasting blood samples and oral glucose tolerance tests (OGTTs) are preferred. Homeostasis model assessment of insulin resistance (HOMA-IR) is calculated as fasting glucose × fasting insulin / 22.5, and the Matsuda index or the composite insulin sensitivity index (ISI) as 10,000 / √(fasting glucose × fasting insulin × mean OGTT glucose × mean OGTT insulin) (DeFronzo & Matsuda, 2010; Matthews et al., 1985).

**Metabolic risks in PCOS**

Dyslipidemia is a common metabolic abnormality in PCOS, although the type and extent of abnormalities may vary. The commonly reported abnormalities include decreased high-density lipoprotein (HDL) levels and elevated low-density lipoprotein (LDL) and triglyceride levels (Diamanti-Kandarakis, Papavassiliou, Kandarakis, & Chrousos, 2007; Hoffman & Ehrmann, 2008; Randeva et al., 2012). An unfavorable lipid profile has been reported in both lean and obese women with
PCOS compared with non-PCOS controls (Glueck, Morrison, Goldenberg, & Wang, 2009; Yildirim, Sabir, & Kaleli, 2003). Women with PCOS are at an increased risk of metabolic syndrome (MetS), a constellation of cardiometabolic risk factors: central obesity, hypertriglyceridemia, low HDL levels, elevated systolic and/or diastolic blood pressure, and elevated fasting glucose (Alberti et al., 2009). The risk of cardiovascular diseases in women with PCOS is not fully understood even though cardiovascular risk factors are considerably increased in such women, these including hyperlipidemia, hypertension, thrombosis and systemic inflammation (M. E. Ollila et al., 2019; Orio, Palomba, & Colao, 2006; Puurunen et al., 2009).

2.1.3 Pathophysiology of PCOS

The pathophysiology of PCOS is complex and multifactorial, and reflects interactions between genetic, fetal, metabolic, and environmental factors. Hyperinsulinemia, disordered gonadotropin secretion, hyperandrogenemia, and ovarian dysfunction are considered prominent factors.

Insulin resistance in the pathogenesis of PCOS

Insulin resistance and compensatory hyperinsulinemia contribute to hyperandrogenemia in PCOS through several mechanisms. Insulin inhibits hepatic SHBG synthesis, leading to increased bioavailability of testosterone (Ehrmann, 2005). Insulin acts synergistically with luteinizing hormone (LH), increasing ovarian androgen synthesis (Diamanti-Kandarakis & Dunaif, 2012; Rosenfield & Ehrmann, 2016). Insulin resistance in PCOS is characterized by selective tissue insulin sensitivity, wherein the metabolic pathway is resistant to insulin action while the steroidogenic pathway seems to be preserved. This is due to the disparate cellular effects of insulin stimulation via phosphatidyl inositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways: the PI3K pathway mediating alterations in glucose metabolism and glucose uptake in skeletal muscle, and the MAPK pathway mediating cell growth and differentiation, and steroidogenic effects (Cusi et al., 2000; Dumesic et al., 2015). It has been suggested that in PCOS, PI3K pathway signaling is disturbed while the MAPK pathway functions normally (Barber et al., 2006). This results in divergent cellular responses to insulin: down-regulated metabolic effects and concurrent augmentation of steroidogenesis,
leading to metabolic dysfunction and hyperandrogenemia (Barber, Dimitriadis, Andreou, & Franks, 2016).

**Hyperandrogenemia**

The origin of hyperandrogenemia in PCOS is multifactorial. Women with PCOS present with a defective hypothalamic-pituitary axis, wherein gonadotropin-releasing hormone (GnRH) pulse frequency from the hypothalamus is increased, leading to increased LH pulse frequency and amplitude, and thus increased serum LH concentrations (Blank, McCartney, & Marshall, 2006). This leads to increased androgen secretion from the ovarian theca cells, which are under LH regulation. Furthermore, in a hyperandrogenic environment, the sensitivity of the GnRH pulse generator to negative feedback by progesterone is impaired (Burt Solorzano et al., 2012). The ovarian theca cells in women with PCOS display an underlying steroidogenic dysfunction, as they are more efficient at converting androgenic precursors into T compared with normal theca cells (Nelson et al., 2001; Rosenfield & Ehrmann, 2016). Although the predominant source of hyperandrogenemia in women with PCOS is ovarian, adrenal androgen secretion is also significant, and seen in 20–30% of women with PCOS (Yildiz & Azziz, 2007). In addition, the peripheral conversion of androstenedione and dehydroepiandrosterone sulfate (DHEAS) also contributes to hyperandrogenemia (Payne & Hales, 2004). Furthermore, IR and compensatory hyperinsulinemia also contribute to hyperandrogenemia. Insulin inhibits hepatic synthesis of SHBG, thus increasing levels of unbound, biologically active T. Also, insulin acts synergistically with LH to increase T production from theca cells (Ehrmann, 2005).

**Genetic component and developmental programming in the pathogenesis of PCOS**

It has been postulated that the syndrome has its origins during fetal programming in the intra-uterine environment. PCOS could be a consequence of glucocorticoid excess due to fetal hypoxia and intrauterine growth restriction (Longo et al., 2013). Animal studies have suggested that early prenatal exposure to androgen excess leads to a PCOS-type phenotype in the offspring, with PCOS-like manifestations further appearing in the second-generation female offspring, suggesting developmental programming *in utero* (Abbott, Barnett, Bruns, & Dumesic, 2005; Dumesic, Abbott, & Padmanabhan, 2007). It has been hypothesized that fetal
androgen excess subsequently reprograms multiple organ systems that will later manifest heterogeneous phenotypes of PCOS (Abbott, Dumesic, & Franks, 2002). Environmental factors, such as endocrine-disrupting chemicals and advanced glycation end products, if persistent, might program reproductive and metabolic functions in females, leading to PCOS (Rutkowska & Diamanti-Kandarakis, 2016). Familial aggregation of PCOS has been recognized, with genome-wide association studies identifying a few genetic variants (McAllister, Legro, Modi, & Strauss, 2015). However, their role in the development of the syndrome is yet to be ascertained (Dumesic et al., 2015).

2.1.4 Adipose tissue dysfunction in PCOS

Adipose tissue is regarded as an endocrine organ that secretes a wide variety of signaling molecules that regulate diverse processes, such as energy expenditure, appetite, glucose homeostasis, insulin sensitivity, inflammation, and tissue repair (Scheja & Heeren, 2019). Adipose tissue in obesity is characterized by hypertrophy of adipocytes, a proinflammatory gene-expression profile, dysregulated secretion of adipokines, increase in macrophages and other immune cells, and a decreased response of adipocytes to insulin (Boucher, Kleinridders, & Kahn, 2014; Lee & Pratley, 2005). Similar, albeit less well characterized changes are seen in the adipose tissue of women with PCOS. Adipocyte diameter in women with PCOS is 25% greater than that seen in obese control women without the syndrome, and hypertrophied adipocytes are strongly associated with IR (Mannerås-Holm et al., 2011).

Adipokines are the bioactive secretory products of adipocytes, altered secretion of which is thought to influence the pathophysiology of PCOS. Adiponectin has direct insulin-sensitizing effects, with downregulation seen in obesity. Decreased serum adiponectin levels seen in women with PCOS may be implicated in the IR associated with the syndrome and could play a possible role in the etiology of PCOS (Toulis et al., 2009). Leptin, an adipokine regulating appetite and energy expenditure, is secreted in direct proportion to the degree of obesity (Budak et al., 2006). Although serum leptin levels in women with PCOS are similar to those in control women, leptin may play a role in the development of PCOS in obese women, as the levels are associated with body-fat percentage (Carmina et al., 2009; Svendsen et al., 2012).

Retinol-binding protein 4 (RBP4), synthesized by hepatocytes and adipose tissue, is the principal transport protein for retinol (vitamin A) in the circulation.
(Blaner, 1989; Blomhoff, Green, Berg, & Norum, 1990). Increased serum RBP4 levels have been reported in insulin-resistant mice and in humans with obesity and T2DM, and subsequently, RBP4 was identified as an adipokine which may contribute to systemic IR (Q. Yang et al., 2005). In mice, overexpression of RBP4 from adipocytes interfered in GLUT4 (glucose transporter type 4 protein) translocation and insulin signaling in skeletal muscle through alterations in PI3K and insulin receptor substrate-1 (IRS-1) activation, leading to abnormal glucose uptake and consequent IR. Elevated RBP4 levels further augmented hepatic gluconeogenesis by inducing phosphoenolpyruvate carboxykinase (PEPCK), a gluconeogenic enzyme (Figure 2) (Q. Yang et al., 2005). In human studies, elevated serum RBP4 concentrations have been reported in both lean and obese subjects with T2DM and IGT, with serum RBP4 levels correlating with the severity of IR (Cho et al., 2006; Graham et al., 2006; Klöting et al., 2007; Norseen et al., 2012). Furthermore, large epidemiologic studies have shown that elevated serum RBP4 levels are associated with components of MetS, including increased BMI, WHR, and serum triglyceride concentrations (Meisinger et al., 2011; Qi et al., 2007). These results suggest that RBP4 could possibly link adiposity with systemic IR in women with PCOS.

Fig. 2. Regulation of glucose metabolism in skeletal muscle and liver through RBP4.
Data regarding serum levels of RBP4 in women with PCOS have been conflicting (Table 3). Increased serum levels of RBP4 have been observed in both lean and overweight/obese women with PCOS (Czeczuga-Semeniuk et al., 2015; Jeon et al., 2013; Weiping et al., 2006). Studies have also shown elevated RBP4 levels only in obese women with PCOS (Yildizhan et al., 2011) or only in lean women with PCOS compared with weight-matched controls (Chan et al., 2010; Diamanti-Kandarakis, Livadas, Kandarakis, Papassotiriou, & Margeli, 2008). Moreover, no differences have been found in RBP4 levels in women with PCOS compared with BMI-matched controls (Barber et al., 2008; Hutchison, Harrison, Stepto, Meyer, & Teede, 2008; Olszanecka-Glinianowicz et al., 2012). Studies regarding the association between serum RBP4 levels and IR in PCOS have also been conflicting; some studies have reported no association (Diamanti-Kandarakis et al., 2008; Hutchison et al., 2008), while others have reported a positive correlation between RBP4 levels and IR, but not PCOS per se (Hahn et al., 2007; Möhlig et al., 2008).

Table 3. Previous studies on RBP4 in women with PCOS.

<table>
<thead>
<tr>
<th>Study</th>
<th>Number of subjects</th>
<th>Age (years)</th>
<th>BMI (kg/m²)</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Czeczuga-Semeniuk et al., 2015</td>
<td>controls=78/PCOS=294</td>
<td>23.2 (1.6)</td>
<td>21.6 (3.1)</td>
<td>RBP4 ↑ in PCOS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24.8 (4.4)</td>
<td>24.7 (5.7)</td>
<td></td>
</tr>
<tr>
<td>Jeon et al., 2013</td>
<td>controls=36/PCOS=54</td>
<td>24.9 (2.9)</td>
<td>19.8 (1.5)</td>
<td>RBP4 ↑ in PCOS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>23.7 (5.3)</td>
<td>23.1 (4.5)</td>
<td></td>
</tr>
<tr>
<td>Olszanecka-Glinianowicz et al., 2012</td>
<td>controls=67/PCOS=83</td>
<td>25.7 (4.9)</td>
<td>28.3 (7.0)</td>
<td>RBP4 ↔ in PCOS and controls</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24.0 (4.8)</td>
<td>29.0 (8.7)</td>
<td>RBP4 ↑ in normal-weight PCOS compared with obese PCOS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>RBP4 inversely correlated with IR</td>
</tr>
<tr>
<td>Yildizhan et al., 2011</td>
<td>controls=27/PCOS=27</td>
<td>25.4 (2.6)</td>
<td>23.9 (3.8)</td>
<td>RBP4 ↑ in obese PCOS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>27.2 (1.7)</td>
<td>30.3 (3.7)</td>
<td>RBP4 does not reflect IR in PCOS</td>
</tr>
<tr>
<td></td>
<td>obese=27/nonobese=30</td>
<td>25.7 (3.7)</td>
<td>23.8 (1.1)</td>
<td></td>
</tr>
<tr>
<td>Chan et al., 2010</td>
<td>controls=37/PCOS=37</td>
<td>29.3 (4.8)</td>
<td>20.8 (2.9)</td>
<td>RBP4 ↑ in PCOS, might arise from triglyceride metabolism</td>
</tr>
<tr>
<td></td>
<td></td>
<td>27.9 (5.2)</td>
<td>22.2 (4.1)</td>
<td>RBP4 levels not influenced by PCOS, not a marker of IR</td>
</tr>
</tbody>
</table>

32
<table>
<thead>
<tr>
<th>Study</th>
<th>Number of subjects</th>
<th>Age (years)</th>
<th>BMI (kg/m²)</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carmina et al., 2009</td>
<td>controls=20, PCOS=48</td>
<td>27.7 (4.5), 27.3 (6.4)</td>
<td>26.8 (3.9), 27.1 (5.6)</td>
<td>RBP4 ↑ in PCOS&lt;br&gt;RBP4 ↑ in ovulatory PCOS compared with anovulatory PCOS despite similar fat distribution</td>
</tr>
<tr>
<td>Huchison et al., 2008</td>
<td>controls=17, PCOS=38</td>
<td>33.2 (1.9), 36.9 (1.4)</td>
<td>34.1 (1.2), 36.8 (1.2)</td>
<td>RBP4 ↔ in PCOS and controls though obese women with PCOS were more IR&lt;br&gt;RBP4 not a marker of IR in PCOS</td>
</tr>
<tr>
<td>Barber et al., 2008</td>
<td>controls=28, PCOS=50</td>
<td>39.5 (6.3), 30.0 (6.6)</td>
<td>28.0, 31.4</td>
<td>RBP4 ↔ in PCOS and controls&lt;br&gt;RBP4 associated with visceral fat, but no primary role in the development of PCOS</td>
</tr>
<tr>
<td>Mohlig M et al., 2008</td>
<td>PCOS=110</td>
<td>28.7 (0.5), 23.0 (4.2)</td>
<td>31.6 (0.8), 26.7 (0.8)</td>
<td>RBP4 ↑ with ↑ IR&lt;br&gt;RBP4 ↑ in women with MetS and IGT/DM</td>
</tr>
<tr>
<td>Diamanti-Kandarakis et al., 2008</td>
<td>controls=45, PCOS=35</td>
<td>25.0 (6.7), 23.0 (4.2)</td>
<td>26.7 (0.8), 28.0 (1.3)</td>
<td>No association with IR</td>
</tr>
<tr>
<td>Hahn et al., 2007</td>
<td>controls=64, PCOS=83</td>
<td>26.1 (5.1), 25.9 (5.5)</td>
<td>21.8 (1.8), 21.9 (1.9)</td>
<td>RBP4 ↔ in lean PCOS and BMI-matched controls&lt;br&gt;RBP4 ↑ in IGT women with PCOS compared with NGT women with PCOS</td>
</tr>
<tr>
<td>Weiping et al., 2006</td>
<td>controls=45, PCOS=39</td>
<td>28.2 (3.1), 27.2 (4.1)</td>
<td>21.2 (2.2), 22.1 (2.6)</td>
<td>RBP4 ↑ in PCOS and associated with IGT/DM</td>
</tr>
</tbody>
</table>

2.1.5 Gastrointestinal permeability and dysbiosis in PCOS

The intestinal epithelium is selectively permeable, allowing absorption of nutrients and water while effectively defending against microorganisms and toxins. Junctional complexes, including tight-junction (TJ) proteins, mediate intestinal permeability (IP) (Suzuki, 2013). Alterations of the intestinal barrier and increased IP, often termed ‘leaky gut’, are thought to play a role in the pathogenesis of various inflammatory and autoimmune disorders (Bischoff et al., 2014; Groschwitz & Hogan, 2009). It has been hypothesized that gut barrier dysfunction is also involved in the pathogenesis of PCOS (Tremellen & Pearce, 2012). Disturbances in the intestinal bacterial flora can cause increased IP, leading to raised lipopolysaccharide passage into the systemic circulation. This triggers a chronic inflammatory response with subsequent IR and increased ovarian androgen production (Tremellen & Pearce, 2012). Furthermore, ovarian theca cells show an altered steroid-hormone response in an inflammatory environment, linking low-grade inflammation to hyperandrogenism (C. W. Fox et al., 2019). In view of the above, altered IP may play a role in the development of PCOS.

Biomarkers of intestinal barrier integrity have been proposed as an alternative to lactulose/rhamnose or lactulose/mannitol urinary excretion ratios, commonly accepted methods of measuring IP (Bischoff et al., 2014; de Kort, Keszthelyi, & Masclee, 2011). Zonulin is a human protein analog of Vibrio cholerae-derived zona occludens toxin and the only known physiological mediator to reversibly regulate IP by modulating intercellular TJs. Zonulin activates TJ disassembly, increasing permeability of the intestinal epithelia and activating immune reactions (Figure 3) (Wang, Uzzau, Goldblum, & Fasano, 2000). Zonulin is regarded as a reliable non-invasive biomarker of IP, as serum levels of zonulin strongly correlate with the lactulose/mannitol ratio (Sapone et al., 2006; Wang et al., 2000). Zonulin levels are increased in obesity, T2DM, gestational diabetes mellitus, and positively correlated with IR and levels of inflammatory markers such as interleukin-6 (Demir et al., 2019; Jayashree et al., 2014; Moreno-Navarrete, Sabater, Ortega, Ricart, & Fernandez-Real, 2012). Conflicting results have been reported as regards serum levels of zonulin, with studies indicating no difference or elevated levels of zonulin in women with PCOS compared with controls (Cetin et al., 2019; D. Zhang, Zhang, Yue, Zheng, & Russell, 2015).
Fatty acid-binding protein 2 (FABP2), also called intestinal (I)-FABP, is an intracellular protein involved in the uptake and metabolism of fatty acids. FABP2 is predominantly expressed in enterocytes of the small intestine. Following mucosal injury of the small intestine, FABP2 is released into the circulation, and elevated levels indicate intestinal epithelial cell damage (D. S. March et al., 2017; Pelsers et al., 2003). Intestinal epithelial cell injury is a key mechanism in altered IP, and FABP2 can be used as an indirect measure of increased IP. Increased FABP2 levels have been reported in severely obese subjects with chronic hyperglycemia (Verdam et al., 2011). Studies have shown that alterations in the gut microbiota are associated with obesity, MetS and T2DM (Tilg & Kaser, 2011; Tilg & Moschen, 2014). Also, it has been suggested that hyperandrogenemia in PCOS may be linked to changes in the gut microbiota, i.e., gut dysbiosis (Thackray, 2019). Bacterial metabolites are released during microbiota growth, which enter the systemic circulation via intestinal absorption and are later excreted in the urine (R. S. Lord & Bralley, 2008). Indican, also known as indoxyl sulfate, is one such metabolite of tryptophan metabolism. Gut microbiota metabolize dietary tryptophan into indole, which after systemic absorption, is sulfated in the liver to indoxyl sulfate, and is
excreted in the urine (Figure 4). Determination of urinary concentrations of indican can be used as a simple measure of assessing dysbiosis.

![Dietary precursor Tryptophan → Indole → Indoxyl sulphate](image)

*Fig. 4. Synthesis of indican.*

### 2.2 Bone metabolism

#### 2.2.1 Bone biology

The skeleton is a specialized tissue with structural, protective and metabolic functions. It provides mechanical support for locomotion, protects vital internal organs and serves as a mineral reservoir to maintain homeostasis. Morphologically, bone can be divided into cortical (compact) and cancellous (trabecular) bone. Cortical bone mainly has structural and load-bearing functions, while cancellous bone is primarily responsible for the metabolic functions of bone (Buckwalter, Glimcher, Cooper, & Recker, 1996). Bone is composed of cells and extracellular matrix with organic and inorganic components. By weight, bone tissue consists of an approximately 70% mineral or inorganic component, 25% organic matrix and 5% water (Sommerfeldt & Rubin, 2001). The inorganic component consists of mainly calcium and phosphate in the form of hydroxyapatite. Type I collagen constitutes approximately 90% of the organic matrix, and the rest is composed of non-collagenous glycoproteins and proteoglycans (Buckwalter *et al.*, 1996). The cell types identified in bone tissue are osteoblasts, osteocytes and bone-lining cells derived from mesenchymal stem cells known as osteoprogenitor cells, and osteoclasts derived from the monocyte/macrophage hematopoietic lineage (Downey & Siegel, 2006). The characteristics and functions of these cells are detailed in Table 4.
### Table 4. Bone cell types and functions.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Morphological characteristics</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteoblasts</td>
<td>cuboidal-shaped with single nucleus tightly packed along the surface linings of bone</td>
<td>synthesis and secretion of extracellular matrix (osteoid) mineralization</td>
</tr>
<tr>
<td>Osteoclasts</td>
<td>multinucleated, polarized cells present on bone surface</td>
<td>bone resorption</td>
</tr>
<tr>
<td>Osteocytes</td>
<td>most abundant, comprising 90−95% of bone cells stellate-shaped, located within the bone matrix</td>
<td>regulation of osteoblast and osteoclast activity mechanosensory cells that coordinate skeletal response to loading regulate mineral homeostasis mineralization of bone extracellular matrix</td>
</tr>
<tr>
<td>Bone-lining cells</td>
<td>flattened cells cover the surface of quiescent bone</td>
<td>regulate bone remodeling</td>
</tr>
</tbody>
</table>

(Buckwalter et al., 1996; Burger & Klein · Nulend, 1999; Everts et al., 2002; Florencio-Silva, Sasso, Sasso-Cerri, Simões, & Cerri, 2015; Teitelbaum, 2000).

#### 2.2.2 Bone remodeling

The skeleton is a dynamic organ that undergoes continuous regeneration through bone modeling and remodeling. Bone modeling is the process where bone formation and resorption occur independently at distinct skeletal sites, resulting in significant changes in bone architecture. In contrast, bone remodeling is the process of bone turnover, where old bone is periodically replaced with newly formed bone at the same site (Manolagas, 2000; Seeman, 2009). Under normal conditions, bone resorption and formation are tightly coupled, so that the overall structure and volume of the bone remains unchanged. In homeostatic equilibrium, each cycle of resorption is followed by formation, so that the quantity of bone removed is always equal to the quantity of newly formed bone (Kenkre & Bassett, 2018; Seibel, 2005). This close coordination between bone resorption and formation allows approximately 10% of the skeleton to be replaced annually while ensuring that the structural integrity of the skeleton is maintained (Manolagas, 2000). Bone remodeling serves to repair fatigue damage of the bone due to repeated loading and to prevent excessive aging and its consequences, thus preserving bone structure, strength and mass (Hadjidakis & Androulakis, 2006).
Bone remodeling is carried out by the coordinated actions of osteoclasts and osteoblasts organized in basic multicellular units that follow an activation-resorption-reversal-formation sequence of events (Hadjidakis & Androulakis, 2006). Remodeling begins with the process of activation and recruitment of osteoclast precursor cells to specific bone surface areas to be remodeled, where they form multinucleated osteoclasts that resorb a pocket of bone (Teitelbaum, 2000). This is followed by the reversal phase during which pre-osteoblasts migrate to the newly resorbed area where they differentiate into osteoblasts. Activated osteoblasts synthesize osteoid in resorption cavities which undergo mineralization after a lag period, and the bone surface returns to its quiescent state (Allen & Burr, 2014; Katsimbri, 2017) (Figure 5).

![Fig. 5. Bone remodeling process.](image)

### 2.2.3 Bone turnover markers

Bone mass is assessed by densitometric techniques such as dual-energy X-ray absorptiometry (DXA) and quantitative computed tomography (QCT), which can be considered as static measures to assess BMD, reflecting long-term net gain or loss of bone mass. In contrast, biochemical markers of bone metabolism, or bone turnover markers (BTMs) can be used to assess the dynamics of metabolic imbalances of bone and skeletal pathology, and they reflect current bone metabolism (Bauer, 2019; Seibel, 2005; Szulc, 2018). BTMs reflect underlying changes in bone mass and are thus predictable of whole-body bone turnover. BTMs have been validated against bone histomorphometry, which is considered to be a gold standard technique for the assessment of bone turnover (Chavassieux, Porto-
Muzy, Roux, Garnero, & Chapurlat, 2015). Bone histomorphometry allows visual assessment of bone formation and resorption in a small part of the skeleton (biopsy from the iliac crest), while BTMs reflect the metabolic status of the whole skeleton (Szulc, 2018). Furthermore, levels of BTMs can measure acute changes in bone turnover, providing a representative view of overall bone loss than that obtained by measurement of BMD at specific skeletal sites (S. D. Vasikaran, 2008). There is a moderate association between baseline BTM levels and subsequent changes in BMD (Ivaska et al., 2008). BTMs are divided into markers of bone formation or resorption.

**Biochemical markers of bone turnover**

- Bone formation markers
  - Propeptides of type I procollagen
    - procollagen type I amino-terminal propeptide (PINP)* (Serum)
    - procollagen type I carboxy-terminal propeptide (PICP) (Serum)
  - Alkaline phosphatase (ALP) (total ALP, bone ALP) (Serum)
  - Osteocalcin (OC) (Serum)
- Bone resorption markers
  - Pyridinoline (PYD) and Deoxypyridinoline (DPD) (Urine)
  - Telopeptides of type I collagen
    - carboxy-terminal cross-linking telopeptide of type I collagen (CTX)* (Serum)
    - amino-terminal cross-linking telopeptide of type I collagen (NTX) (Urine)
    - carboxy-terminal cross-linking telopeptide of type I collagen generated by metalloproteinases (ICTP/CTX-MMP) (Serum)
  - Tartrate-resistant acid phosphatase (TRAP) (Serum)

*Recommended by the International Osteoporosis Foundation (IOF) and the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) as reference analytes in clinical and research studies (S. Vasikaran et al., 2011).

**Markers of bone formation**

Bone formation markers are products of osteoblasts expressed during different phases of their development and they reflect various aspects of osteoblast function.
Propeptides of type I collagen

Type I collagen molecule, a triple helix of two α1(I)- and one α2(I) chains, is synthesized as a precursor, type I procollagen, which contains additional domains at both ends of the procollagen molecule (Figure 6). These extensions, known as propeptides, are enzymatically cleaved from procollagen in the extracellular space to form procollagen type I carboxy-terminal propeptide (PICP) and procollagen type I amino-terminal propeptide (PINP). These propeptides are cleared from the circulation by hepatic endothelial cells (Seibel, 2000; Szulc, 2018). The International Osteoporosis Foundation (IOF) and the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) have recommended serum PINP as the reference marker for bone formation (S. Vasikaran et al., 2011).

Fig. 6. Type I procollagen molecule with domains.

Osteocalcin

Osteocalcin (OC) is a hydroxyapatite-binding protein synthesized mainly by osteoblasts. It contains three vitamin K-dependent γ-carboxy glutamic acid residues responsible for the calcium-binding properties of the protein (J. Li, Zhang, Yang, Li, & Dai, 2016). After its release, a major part of OC is incorporated into the extracellular bone matrix, while the smaller part is released into the circulation. Osteocalcin is considered to be a specific marker of osteoblast function, as its levels correlate well with bone-formation rates (Mizokami, Kawakubo-Yasukochi, & Hirata, 2017). However, the peptide undergoes rapid degradation in serum, and both intact peptide and OC fragments of different sizes are found in the circulation (Delmas et al., 2000).
Alkaline phosphatase

Alkaline phosphatase (ALP) plays a vital role in osteoid formation and mineralization. The total ALP serum pool consists of several isoenzymes derived from liver, bone, intestine, spleen, kidney, and placenta. About 50% of the total ALP activity in serum is derived from the liver, while rest arises from bone. Bone alkaline phosphatase (BALP) is bone-specific, but it shows 20% cross-reactivity with the liver isoform (Delmas et al., 2000; Woitge & Seibel, 2017).

Markers of bone resorption

Pyridinoline (PYD) and deoxypyridinoline (DPD)

Collagen fibrils are strengthened by amino acids forming crosslinks between the fibrils. PYD and DPD, the pyridinium crosslinks found in the fibril-forming collagen types, are released into the circulation as degradation products of mature collagen. Bone is the major contributing source of DPD in the circulation, while both bone and cartilage contribute to PYD. PYD and DPD crosslinks are biomarkers of collagen degradation that can be measured in urine (Seibel, 2000; Szulc, 2018).

Telopeptides of type I collagen

Type I collagen telopeptides are released into the circulation during bone resorption. These include the carboxy-terminal cross-linking telopeptide of type I collagen (CTX) and the amino-terminal cross-linking telopeptide of type I collagen (NTX) generated by cathepsin K, and the carboxy-terminal cross-linking telopeptide of type I collagen generated by matrix metalloproteinases (CTX-MMP, also called ICTP) (Delmas et al., 2000; Szulc, 2018). The IOF and the IFCC have recommended serum CTX as the reference marker for bone resorption (S. Vasikaran et al., 2011).

Tartrate-resistant acid phosphatase (TRAP)

TRAP exists in two isoforms; 5a expressed by macrophages and 5b, characteristic of bone resorbing osteoclasts. Osteoclasts contain high amounts of TRAP-5b which is secreted into the circulation. Circulating TRAP-5b is derived exclusively from
osteoclasts. Furthermore, TRAP-5b levels reflect osteoclast numbers and activity (Halleen, 2003; Henriksen et al., 2007).

Sources of variability and clinical utility of BTMs

Measurements of BTMs are influenced by several controllable and uncontrollable factors, which affect pre-analytical variability. The controllable factors having a major influence on the variability of BTM levels include circadian rhythm and food intake, which is particularly pronounced as regards bone resorption markers (Szulc, 2018). CTX levels peak at night-time, falling to a nadir in the middle of the day, and decreased levels are seen after food intake. Hence, sample collection in the morning after an overnight fast is recommended for CTX measurements (Szulc et al., 2017). Other controllable factors having moderate to minimal impacts include exercise, menstrual cycle and season (Szulc, 2018; S. Vasikaran et al., 2011). The uncontrollable factors with a major effect on BTM levels include age, menopausal status, and gender. Levels of BTMs increase with age and within a few months after the last menstrual period. Higher levels are seen in older women than older men (Szulc, 2018; S. Vasikaran et al., 2011). Other important non-modifiable factor includes a recent fracture, wherein a peak increase occurs at 1–3 months after fracture with levels returning to the pre-fracture state by one year (Naylor & Eastell, 2012).

Peak bone mass

Peak bone mass, the maximal amount of bone gained at the end of skeletal growth, is an important predictor of osteoporosis later in life (Weaver et al., 2016). Several factors, including genetic, endocrine, nutritional and mechanical factors, influence peak bone mass (Bonjour, Chevalley, Ferrari, & Rizzoli, 2012). Estrogen is the important gonadal sex-steroid involved in the maturation and maintenance of skeletal mass (Soyka, Fairfield, & Klibanski, 2000). Estrogen, through effects mediated by estrogen receptors in bone cells, inhibits osteoclast activity, and increases osteoclast apoptosis and osteoblast differentiation. This suppresses bone resorption, with increased bone formation, leading to a marked increase in BMD starting at menarche, with peak bone density by the age of 25–35 years in women (Clarke & Khosla, 2010). In addition, estrogen stimulates other mediators of skeletal growth such as growth hormone (GH) and insulin-like growth factor 1 (IGF-1). Most of the GH action on bone is mediated through IGF-1, which acts as
a bone trophic hormone by increasing osteoblastic activity and bone formation (Soyka et al., 2000).

2.3 PCOS and bone health

PCOS is characterized by changes in several hormonal and metabolic systems, which can affect bone metabolism and skeletal mass. Furthermore, menstrual dysfunction during the attainment of peak skeletal mass, from late adolescence to the early thirties, may also influence bone mass accrual (Chew & Clarke, 2018; Recker et al., 1992; Weaver et al., 2016). Studies on BMD in women with PCOS, assessed by DXA, have shown conflicting results; no difference in BMD (Adami et al., 1998; Berberoglu et al., 2015; Carmina, Guastella, Longo, Rini, & Lobo, 2009; S. Gao, Cheng, Zhao, Chen, & Liu, 2016; Glintborg, Andersen, Hagen, Heickendorff, & Hermann, 2008; Kalyan et al., 2017; Noyan, Yucel, & Sagsoz, 2004), decreased BMD (Karadag et al., 2017; Katulski et al., 2014; Yüksel, Dökmetas, Topcu, Erselcan, & Sencan, 2001) and increased BMD (Di Carlo et al., 1992) have been reported compared with control women. Also, the long-term impact of PCOS on skeletal health is inconclusive, as both increased and decreased fracture risk has been reported (Rubin et al., 2016; H. Y. Yang et al., 2018).

2.3.1 Effect of metabolic alterations in PCOS on bone metabolism

Androgens have direct and indirect effects on bone. The primary mechanism of androgen action on bone metabolism is thought to be the aromatization of androgens to estrogens in the ovary and extra-ovarian tissues, with subsequent binding to estrogen receptors in target tissues (Clarke & Khosla, 2009; Notelovitz, 2002). Estrogen plays a crucial role in maintaining bone mass in women (Khosla & Monroe, 2018; Riggs, Khosla, & Melton, 2002). The presence of androgen receptors on osteoblasts indicates a direct effect of androgens on bone (Chen, Lin, Tsai, Yang, & Kang, 2019; Vanderschueren et al., 2004). It has also been suggested that androgenic hormones may stimulate bone formation by the induction of type I collagen synthesis. Androgens, the bioavailability of which can be modified by circulating SHBG levels, might play a role in determining bone mass in premenopausal women (Zborowski, Cauley, Talbott, Guzick, & Winters, 2000). Free T has been consistently associated with BMD in premenopausal women (Notelovitz, 2002). Dehydroepiandrosterone (DHEA) directly stimulates osteoblast proliferation and differentiation. Less potent DHEAS is unlikely to have
direct stimulatory effects on bone and serves as a surrogate marker of DHEA effects. Conversely, DHEAS may exert its influence through estrogen receptors by metabolizing to estrogens (Kirby, Buchalter, Anil, & Leucht, 2020; Krishnan & Muthusami, 2017). Eumenorrheic women with PCOS, when compared with non-eumenorrheic women with PCOS or controls, display increased bone density, suggesting that both estrogens and androgens have a critical role in maintaining bone mass, and androgens have a positive effect on bone only in the presence of adequate estrogens (Zborowski et al., 2000). However, not all studies have shown increased bone density in women with PCOS (Berberoglu et al., 2015; Kirchengast & Huber, 2001; Yüksel et al., 2001).

Elevated insulin levels have direct stimulatory effects on osteoblastic activity, promoting the proliferation and differentiation of osteoblasts (N. Zhang et al., 2019). In addition, insulin suppresses insulin-like growth factor (IGF) binding-proteins in bone cells. This insulin-induced suppression of binding-proteins may lead to increased exposure of target tissues, including bone, to high levels of IGFs (Thrailkill, Lumpkin, Bunn, Kemp, & Fowlkes, 2005). Thus, hyperinsulinemia might positively affect bone density (Krishnan & Muthusami, 2017). Obesity, a common feature among women with PCOS, affects bone metabolism through several mechanisms. Mechanical loading, conferred by body weight, stimulates bone formation by decreasing apoptosis and increasing proliferation and differentiation of osteoblasts and osteocytes (Cao, 2011; Ehrlich & Lanyon, 2002). Adipocytes and stromal cells in adipose tissue express P450 aromatase, which converts adrenal and ovarian androgens to 17β-estradiol and estrone (S. Patel, 2017; Zborowski et al., 2000). As mentioned earlier, estrogen has a critical role in maintaining bone mass in women.

The main physiological role of vitamin D is the regulation of calcium homeostasis, promoting bone health. Vitamin D deficiency adversely affects bone mineralization, bone remodeling and BMD (Garnero, Munoz, Sornay-Rendu, & Delmas, 2007). The serum concentration of 25-hydroxyvitamin D (25OHD) is considered to be the most reliable measure to evaluate vitamin D status. According to Endocrine Society Clinical Practice guidelines, vitamin D deficiency is defined as a concentration of 25OHD below 50 nmol/L, and vitamin D insufficiency as a level of 25OHD of 52.5–72.5 nmol/L (Holick et al., 2011). Studies have reported significantly lower levels of 25OHD in women with PCOS compared with controls (Bacopoulou, Kolias, Efthymiou, Antonopoulos, & Charmandari, 2017; Hahn et al., 2006; H. W. Li, Brereton, Anderson, Wallace, & Ho, 2011). The reported
prevalence of vitamin D deficiency in women with PCOS is between 67−85% (Thomson, Spedding, & Buckley, 2012).

### 2.3.2 Bone turnover markers in PCOS

Limited albeit conflicting results have been reported on BTMs in women with PCOS (Table 5). In all studies, except one, OC was used as a marker of bone formation, whereas data on resorption markers, if at all measured, was not consistent between the studies (Adami et al., 1998; Berberoglu et al., 2015; Diamanti-Kandarakis et al., 2011; Glintborg et al., 2008; Pepene, 2013). Only one study used the BTMs recommended by the IOF and the IFCC (S. Gao et al., 2016).

#### Table 5. Studies on bone turnover markers in women with PCOS.

<table>
<thead>
<tr>
<th>Study</th>
<th>Number of subjects</th>
<th>Age (years)</th>
<th>BMI (kg/m²)</th>
<th>Markers measured</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gao et al., 2016</td>
<td>controls−39/PCOS−52</td>
<td>22.5 (5.3)</td>
<td>22.9 (3.5)</td>
<td>Formation: PINP</td>
<td>↔ BTMs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>26.0 (3.2)</td>
<td>26.6 (5.6)</td>
<td>Resorption: CTX</td>
<td></td>
</tr>
<tr>
<td>Berberoglu Z et al., 2015</td>
<td>controls−20/PCOS−42</td>
<td>30.9 (4.1)</td>
<td>36.4 (4.2)</td>
<td>Formation: BALP, OC</td>
<td>↔ BTMs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>29.6 (3.7)</td>
<td>36.5 (3.7)</td>
<td>Resorption: DPD, PYD</td>
<td></td>
</tr>
<tr>
<td>Pepene et al., 2013</td>
<td>controls−26/PCOS−52</td>
<td>26.0 (7.3)</td>
<td>27.0 (6.0)</td>
<td>Formation: OC</td>
<td>↔ OC</td>
</tr>
<tr>
<td>Diamanti-Kandarakis et al., 2011</td>
<td>controls−47/PCOS−50</td>
<td>27.2 (6.7)</td>
<td>26.3 (5.3)</td>
<td>Formation: OC</td>
<td>↓ OC in PCOS</td>
</tr>
<tr>
<td>Glintborg et al., 2008</td>
<td>controls−14/PCOS−30</td>
<td>36 (14.3)</td>
<td>33.2 (8.2)</td>
<td>Formation: ALP, OC</td>
<td>↔ ALP and OC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>33 (14.0)</td>
<td>33.0 (7.6)</td>
<td>Resorption: ICTP</td>
<td>↑ ICTP in PCOS</td>
</tr>
<tr>
<td>Adami et al., 1998</td>
<td>controls−35/PCOS−51</td>
<td>26.0 (6.5)</td>
<td>21.7 (3.2)</td>
<td>Formation: OC</td>
<td>↔ BTMs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24.2 (4.9)</td>
<td>23.5 (4.8)</td>
<td>Resorption: DPD, hydroxyproline</td>
<td></td>
</tr>
</tbody>
</table>

2.3.3 Metformin and bone metabolism

Metformin is the reference off-label drug for the treatment of PCOS worldwide. It acts by inhibiting hepatic glucose production and increasing peripheral tissue sensitivity to insulin (Viollet et al., 2012). Studies have reported that long-term treatment with metformin regularizes menstrual cyclicity, normalizes ovulation and hyperandrogenemia (Diamanti-Kandarakis, Christakou, Kandarakis, & Economou, 2010). Metformin lowers hepatic glucose production through activation of the 5’ adenosine monophosphate-activated protein kinase (AMPK) pathway (Zhou et al., 2001). Furthermore, AMPK subunits are highly expressed on osteoblasts and osteoclasts (Tong, Ganta, & Liu, 2020). Cellular and animal studies have shown conflicting results on the effect of metformin on bone metabolism. The former have shown that metformin is a potent stimulator of AMPK activation in osteoblasts, resulting in their differentiation and mineralization (Y. Gao, Li, Xue, Jia, & Hu, 2010; Mai et al., 2011). Further, metformin stimulates type I collagen production in osteoblast-like cell lines, suggesting a direct osteogenic effect (Cortizo, Sedlinsky, McCarthy, Blanco, & Schurman, 2006; Kanazawa, Yamaguchi, Yano, Yamauchi, & Sugimoto, 2008; Molinuevo et al., 2010). In contrast, some other studies have not shown such an effect (Kasai et al., 2009; Wu, Ye, Zhou, & Tan, 2011). Metformin treatment prevents bone loss in ovariectomized rats, suggesting a protective action of metformin against bone loss (Y. Gao et al., 2010; Mai et al., 2011). Conversely, it has also been reported that metformin has no effect on bone mass in rodents (Jeyabalan et al., 2013). Though a widely used drug, no studies have been carried out to evaluate the effect of metformin on BMD or BTMs in adult PCOS populations. In one study on adolescents, with a mean age of 15 years, BMD was unchanged after 1.9 years of metformin treatment, but no BTMs were evaluated (Bechtold et al., 2012). It has been reported that metformin decreases fracture risk in patients with T2DM, whereas another study showed no association between metformin and fracture incidents (Melton, Leibson, Achenbach, Therneau, & Khosla, 2008; Monami et al., 2008).
3 Purpose of the present study

Polycystic ovary syndrome is heterogeneous syndrome comprising reproductive, metabolic and hormonal disturbances. Adipose tissue dysfunction, via the secretion of adipocytokines, including retinol-binding protein 4, is thought to play a significant role in the metabolic abnormalities observed in women with PCOS. Furthermore, it has been hypothesized that altered intestinal permeability influences the pathogenesis of the syndrome. Several components of the syndrome, including hyperandrogenemia and hyperinsulinemia, have implications on bone metabolism. The purpose of the study was to determine the potential influence of PCOS on bone turnover and the roles of adipokines and intestinal permeability in the pathogenesis of the syndrome.

The specific aims of the study were:

1. To assess the relationship between PCOS and bone metabolism by determining bone turnover markers (BTMs) and studying the effect of metformin, an insulin-sensitizing drug used in the management of PCOS, on bone turnover in women with the syndrome (Studies I and II)
2. To study the relationship between PCOS and serum retinol-binding protein 4 (RBP4), an adipokine affecting systemic insulin sensitivity and glucose homeostasis (Study III)
3. To study markers of gastrointestinal permeability and dysbiosis in PCOS (Study IV)
4 Subjects and methods

4.1 Study population

4.1.1 Nordic multicenter data (Studies I and III)

Study I and Study III included subjects from six Nordic studies (four studies in Finland and two in Sweden), with 278–298 women with PCOS (age range 18–57 years) and 191–194 non-PCOS control women (age range 20–53 years) (Hudecova, Holte, Olovsson, & Sundstrom Poromaa, 2009; L. Morin-Papunen et al., 2012; Piltonen et al., 2012; Puurunen et al., 2009; Puurunen et al., 2011; Puurunen et al., 2013; Stener-Victorin et al., 2010). The primary studies were conducted during 2003–2011.

PCOS was diagnosed according to the ESHRE/ASRM consensus definition (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004). Ovarian morphology was assessed by means of transvaginal ultrasonography in all subjects. Biochemical hyperandrogenism was defined as a serum testosterone concentration of ≥ 2.3 nmol/L, according to the upper limits of the accredited laboratory NordLab (formerly Oulu University Hospital laboratory) in fertile-aged women, and clinical hyperandrogenism (hirsutism) was diagnosed when a subject had an FG score of > 7. The control population consisted of women with normal-appearing ovaries in ultrasonography and absence of PCOS-related symptoms and findings, e.g. oligo- or amenorrhea and/or hirsutism and/or elevated serum testosterone levels. Women using hormonal preparations and/or medication affecting bone or glucose metabolism, or steroid synthesis were excluded from the study. Alternatively, a washout period of at least two months was required among women using hormonal preparations prior to participation in the studies. All samples were collected in a fasting state.

4.1.2 Randomized clinical trial data (Study II)

Study II included 118 women with PCOS (age range 18–38 years), selected from a cohort of a multicenter, randomized, placebo-controlled study on the effects of metformin on miscarriage, pregnancy and live-birth rates (L. Morin-Papunen et al., 2012). All women included in Study II were examined and recruited at Oulu University Hospital during 2003–2009, and PCOS was diagnosed according to the
ESHRE/ASRM consensus definition as described above. Women who became pregnant or had a miscarriage before the study period of three months were excluded from the study. The study subjects were not on medications known to affect hormonal or metabolic parameters or bone metabolism, and they did not have a history of fracture in the preceding six months.

The subjects were randomized to receive metformin (Diformin; Leiras) or placebo for three months. Non-obese women (BMI < 27 kg/m²) received metformin at a dose of 500 mg + 1000 mg daily and obese women (BMI ≥ 27 kg/m²) received metformin at 1000 mg × 2 daily. The limit for BMI was chosen on the basis of earlier studies showing increased insulin resistance in women with PCOS at a BMI of 27 kg/m² (Gennarelli et al., 2000). The dosage of metformin was based on previous studies, which showed that 1500 mg and 2000 mg of metformin was effective in restoring ovulation in most of the non-obese and obese women with PCOS, respectively, and it improved hyperandrogenism and insulin sensitivity (L. Morin-Papunen et al., 2003; L. C. Morin-Papunen et al., 2000; Nestler & Jakubowicz, 1997).

Clinical, hormonal and metabolic parameters were examined 1−7 days after spontaneous menstruation in oligomenorrheic subjects and at any convenient time in amenorrheic women. A second examination was scheduled three months after the first visit. Fifty-seven women (17 obese, 40 non-obese) received metformin, and 61 (27 obese, 34 non-obese) received placebo for three months. Blood samples were collected in a fasting state at baseline and at three months of treatment with metformin/placebo, the treatment being ongoing at the time of sample collection. Phenotypic characteristics and clinical, hormonal and metabolic parameters of the study population are shown in Tables 6 and 7, respectively.

Table 6. Phenotypic characteristics of the randomized PCOS study population.

<table>
<thead>
<tr>
<th>Phenotypic characteristic</th>
<th>All subjects (n=118)</th>
<th>Metformin (n=57)</th>
<th>Placebo (n=61)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCO + OA + HA</td>
<td>37 (31%)</td>
<td>21 (37%)</td>
<td>16 (26%)</td>
</tr>
<tr>
<td>PCO + OA</td>
<td>79 (67%)</td>
<td>35 (61%)</td>
<td>44 (72%)</td>
</tr>
<tr>
<td>PCO + HA</td>
<td>2 (2%)</td>
<td>1 (2%)</td>
<td>1 (2%)</td>
</tr>
</tbody>
</table>

Table 7. Clinical, hormonal, and metabolic parameters of subjects at baseline in Study II.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Non-obese (BMI &lt; 27 kg/m²)</th>
<th>Obese (BMI ≥ 27 kg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Metformin</td>
<td>Placebo</td>
</tr>
<tr>
<td>Number of subjects</td>
<td>40</td>
<td>34</td>
</tr>
<tr>
<td>Age (years)</td>
<td>27.1 (3.1)</td>
<td>27.9 (4.2)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.5 (2.2)</td>
<td>22.7 (2.6)</td>
</tr>
<tr>
<td>WHR</td>
<td>0.76 (0.05)</td>
<td>0.78 (0.06)</td>
</tr>
<tr>
<td>Hirsutism score</td>
<td>4.8 (3.1)</td>
<td>4.9 (3.2)</td>
</tr>
<tr>
<td>T (nmol/L)</td>
<td>1.6 (0.7)</td>
<td>1.7 (0.7)</td>
</tr>
<tr>
<td>SHBG (nmol/L)</td>
<td>59.0 (20.2)</td>
<td>56.6 (18.1)</td>
</tr>
<tr>
<td>FAI</td>
<td>3.0 (1.9)</td>
<td>3.3 (2.2)</td>
</tr>
<tr>
<td>DHEAS (µmol/L)</td>
<td>5.3 (2.3)</td>
<td>6.2 (3.3)</td>
</tr>
<tr>
<td>Androstenedione (nmol/L)</td>
<td>17.8 (9.5)</td>
<td>21.4 (7.7)</td>
</tr>
<tr>
<td>Estradiol (pmol/L)</td>
<td>209.0 (93.8)</td>
<td>231.2 (98.4)</td>
</tr>
<tr>
<td>Fasting glucose (mmol/L)</td>
<td>5.0 (0.5)</td>
<td>5.1 (0.4)</td>
</tr>
<tr>
<td>Fasting insulin (mU/L)</td>
<td>5.6 (3.1)</td>
<td>6.4 (2.8)</td>
</tr>
</tbody>
</table>

BMI: body mass index, WHR: waist-hip ratio, T: testosterone, SHBG: sex hormone-binding globulin, FAI: free androgen index, DHEAS: dehydroepiandrosterone sulfate. Data shown as mean (SD).

4.1.3 Northern Finland Birth Cohort (NFBC) 1966 data (Study IV)

Study IV included 104 women with PCOS and 203 non-PCOS control women at age 46. Two BMI-matched control women were chosen for each woman with PCOS. The study population was a subset of women from the prospective, longitudinal population-based NFBC1966, which comprises all individuals born alive in 1966 in the two northernmost provinces of Finland. At age 31, a comprehensive postal questionnaire was sent to all women traceable. In addition, most of the women participated in clinical examinations including anthropometric measurements and blood sampling. At age 46, all women with known addresses were invited to take part in follow-up through postal questionnaires. Also, most of the women again participated in clinical examinations. Based on the questionnaires at ages 31 and 46, women with PCOS in the cohort were identified.

At age 31, the questionnaire included two questions on PCOS symptoms: (i) is your menstrual cycle often (more than twice a year) longer than 35 days (oligomenorrhea, OA)? and (ii) do you have bothersome, excessive body-hair growth (hirsutism, H)?” Women were considered as PCOS cases if they answered “yes” to both the questions. At age 46, the questionnaire included the question “Have you ever been diagnosed with polycystic ovaries and/or PCOS?” Women
were considered as PCOS cases if they answered “yes.” Consequently, PCOS was diagnosed in women reporting OA+H at age 31 and/or having a diagnosis of PCOS by age 46. The validity of the questionnaires in identifying women with PCOS has been documented in previous publications concerning the same cohort (Karjula 2017, Koivuaho 2019, Ollila MM 2016, Taponen 2003, Taponen 2004). The control population included women who fulfilled both criteria of no PCOS symptoms (OA+H) at age 31 and without a PCOS diagnosis by age 46. Women using hormonal contraceptives and pregnant women, and those not permitting the use of their data were excluded. The study flowchart is shown in Figure 7.

Fig. 7. Flowchart of the study population in Study IV.

The Ethics Committee of the Northern Ostrobothnia Hospital District in Oulu, Finland, approved the studies.
4.2 Methods

4.2.1 Anthropometric measurements

Body mass index (BMI) was calculated as the ratio of weight (kg) to height squared (m²). Waist circumference was measured at the level midway between the lowest rib margin and the iliac crest, and hip circumference was measured at the widest part of the gluteal region. The waist-hip ratio (WHR) was calculated as the ratio of waist circumference (cm) to hip circumference (cm).

4.2.2 Laboratory analyses

Serum concentrations of PINP, CTX, 25OHD (Studies I and II) and OC (Study I) were measured by automated chemiluminescence immunoassays (CLIAs) using IDS-iSYS Multi-Discipline Automated Analyzer (Immunodiagnostics Systems, Boldon, UK) according to the manufacturer’s protocol. The assays are based on chemiluminescence technology (Cinquanta, Fontana, & Bizzaro, 2017). In brief, samples are incubated with the corresponding monoclonal antibodies labeled with biotin and acridinium. Following the addition of streptavidin-coated magnetic particles and further incubation, the particles are captured using a magnet and a wash step performed. Trigger reagents are added, and the resulting light emitted by the acridinium label is directly proportional to the concentration of the analyte in the sample. The detectable ranges of the assays for PINP, CTX, OC and 25OHD were 2–230 µg/L, 0.033–6 µg/L, 2–200 µg/L and 5–140 µg/L respectively. The respective intra- and inter-assay coefficients of variation (CVs) were 7.6% and 7.3% for PINP, 3.1% and 6.2% for CTX, 4.4% and 3% for OC, and 5.1% and 14% for 25OHD.

Serum concentrations of RBP4 (Study III), FABP2 and zonulin (Study IV) were measured by enzyme-linked immunosorbent assays (ELISAs) according to the manufacturer’s instructions (RBP4 and zonulin: R&D Systems, Minneapolis, USA; FABP2: Immundiagnostik AG, Bensheim, Germany). The intra- and inter-assay CVs were 6.5 and 9% for RBP4, 4.1% and 11.1% for FABP2, and 6% and 13.6% for zonulin. Urinary concentrations of indican (Study IV) were measured by colorimetric assay, according to the instructions of the manufacturer (Sigma-Aldrich, MO, USA), with a detection range between 0.2–20 mg/dL.

In Studies I–IV, serum T concentrations were analyzed using Agilent triple quadrupole 6410 liquid chromatography–mass spectrometry equipment with an
electrospray ionization source operating in positive-ion mode (Agilent Technologies, Wilmington, DE, USA). Multiple reaction monitoring was used to quantify T by trideuterated T. Intra-assay coefficients of variation of the method were 5.3%, 1.6%, and 1.2% for T at 0.6, 6.6, and 27.7 nmol/L, respectively. Inter-assay coefficients of variation were 5.3%, 4.2%, and 1.0% for the respective concentrations. Serum SHBG concentrations were analyzed by chemiluminometric immunoassay (Immulite 2000, Siemens Healthcare Diagnostics, Llanberis, UK). The FAI was calculated as T/SHBG (both as nmol/L) × 100. Androstenedione and DHEAS (Studies I–III) were analyzed in the laboratories of different study sites according to their routine methods (immunoassay and mass spectrometry).

In Study II, plasma glucose was determined by chemical analyzer (Advia 1800, Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA) and serum insulin by chemiluminescent immunoassay (Immulite 2000, Siemens Healthcare, Llanberis, UK). In Studies III and IV, serum levels of glucose, total cholesterol, HDL, LDL, and triglycerides were determined by using an automatic chemical analyzer (Advia 1800, Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA), insulin by an automated chemiluminescence system (Advia Centaur XP, Siemens Healthcare Diagnostics, Tarrytown, NY, USA), and high-sensitivity C-reactive protein (hs-CRP) by immunonephelometric assay (BN ProSpec, Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA).

Oral glucose tolerance tests were carried out after an overnight fast (Studies II–IV). Plasma glucose and serum insulin were measured at baseline and at 30, 60 and 120 min after a 75-g glucose intake. Mean OGTT plasma glucose and serum insulin levels were calculated as the means of concentrations at two different time points [(basal + 2-hour)/2]. Insulin resistance was defined by HOMA-IR and insulin sensitivity by composite ISI or the Matsuda index (DeFronzo & Matsuda, 2010; Matthews et al., 1985). Glucose tolerance was classified according to American Diabetes Association (ADA) guidelines as normal glucose tolerance (NGT) [fasting glucose ≤ 5.5 mmol/L and 2h OGTT glucose < 7.8 mmol/L], impaired fasting glucose (IFG) [fasting glucose 5.6–6.9 mmol/L and normal 2h OGTT glucose], and impaired glucose tolerance (IGT) [normal fasting glucose and 2h OGTT glucose 7.8–11.0 mmol/L].
4.2.3 Statistical methods

Statistical analysis was carried out by using SPSS software (IBM Corp., Armonk, NY) versions 22.0 (Study I) and 25.0 (Studies II–IV). Variables with a skewed distribution were logarithmically transformed before statistical analysis. A value of $p < 0.05$ was considered statistically significant.

Differences between two study groups were assessed using independent-samples $t$-tests (Studies I–IV), and paired-samples $t$-tests were used to evaluate changes between measurements at baseline and after three months of treatment within the groups (Study II). Adjustment for age and BMI was carried out by means of univariate analysis of variance using age and BMI as covariates. The PCOS and control populations were grouped according to age and BMI as follows: ≤ 30 years, 31–40 years and 41 years to menopause (Studies I and III), and normal weight (BMI < 25 kg/m²), overweight (25–30 kg/m²) and obese (> 30 kg/m²) (Study IV). One-way analysis of variance (ANOVA) was used to assess age- and BMI-related changes (Studies III and IV). General linear modeling was used to evaluate the significant determinant of changes in BTM levels (Study II). Correlations between different parameters were assessed by Pearson's and Spearman's correlation coefficients, and adjustment for BMI was carried out by partial correlation analysis. The lowest and highest quartiles were calculated for gut permeability markers (Study IV), and clinical parameters of subjects compared in each quartile.
Table 8. Subject characteristics, study parameters and main results of Studies I–IV.

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5 Results and discussion

5.1 Bone metabolism and effect of metformin on bone turnover in PCOS (Studies I and II)

The aim of Study I was to investigate the relationship between PCOS and bone metabolism by determining cross-sectionally the concentrations of selected BTMs in women with PCOS and control women. Further, age-related changes of BTMs and their associations with metabolic parameters in women with PCOS and controls were evaluated. Concentrations of the bone formation markers PINP and OC were decreased significantly, even after age- and BMI-adjustment, in women with PCOS compared with the controls, while levels of the bone resorption marker CTX were similar. Age-stratified analysis further demonstrated that these changes were seen only in women with PCOS aged ≤ 30 years. Concentrations of the bone formation markers PINP and OC and the resorption marker CTX decreased with age until menopause in both control women and women with PCOS (Figure 8).

Fig. 8. Concentrations (mean ± SEM) of serum bone turnover markers in control women and women with polycystic ovary syndrome stratified according to age. PINP: procollagen type I amino-terminal propeptide, OC: osteocalcin, CTX: carboxy-terminal cross-linking telopeptide of type I collagen, SEM: standard error of the mean. ***BMI-adjusted \( p < 0.001 \). Number of controls vs. PCOS at ≤ 30 y, 31–40 y and 41–menopause: 92 vs. 160, 54 vs. 75 and 48 vs. 63, respectively.

The findings of Study I demonstrated that bone formation is lower in women with PCOS aged ≤ 30 years, while bone resorption is unaffected. Earlier studies have reported conflicting results as regards BTMs in women with PCOS, with varying use of formation and resorption markers and a limited number of study subjects (Table 5). The similar levels of BTMs in women with PCOS compared with controls, as reported in most of the studies, could be due to a relatively small
number of study subjects. The IOF and the IFCC have recommended PINP and CTX as reference analytes in clinical and research studies in order to achieve uniformity in the measurement of BTMs (S. Vasikaran et al., 2011). In contrast to our results, an earlier study showed similar levels of PINP in women with PCOS with a mean age of 22.5 years when compared with controls (S. Gao et al., 2016). A recent meta-analysis, where the mean age of women included ranged from 22.5 to 47.5 years, reported decreased bone formation, as manifested by lower levels of OC, in women with PCOS with a BMI of < 27 kg/m² (Piovezan, Premaor, & Comim, 2019).

Baseline features of women with PCOS aged ≤ 30 years showed that the levels of T, FAI and androstenedione were significantly higher compared with controls of the same age group (Figure 9). However, BMI-adjusted correlation analyses revealed no significant associations between androgens and BTMs, suggesting that decreased bone formation in women with PCOS under 30 years of age may not be associated with altered androgen levels in these women.

Serum levels of 25OHD were similar in age-stratified women with PCOS and controls, and no significant associations were found between BTMs and 25OHD, suggesting that 25OHD may not have a major influence on bone turnover. This observation is supported by the results of earlier studies showing that 25OHD does not significantly influence the concentrations of BTMs (de Papp et al., 2007; Garnero, Sornay-Rendu, Chapuy, & Delmas, 1996).

In conclusion, women with PCOS under the age of 30 had decreased circulating levels of the bone formation markers PINP and OC, and unchanged levels of the bone resorption marker CTX, compared with age-matched controls. These findings suggest that bone formation is decreased in women with PCOS under the age of 30, with a possible negative impact on bone health in these women. However, since single time-point measurements were done, and longer follow-up data on BTMs was not available in these women, long-term conclusions cannot be inferred. Moreover, it has to be noted that BTMs alone have limited predictive value as regards the incidence of fractures, considered as the primary end point where bone health is concerned (Glendenning, Chubb, & Vasikaran, 2018; Schmidt, Dahlgren, Brännström, & Landin-Wilhelmsen, 2012; Vilaca, Gossiel, & Eastell, 2017). The results of our study have to be further validated in long-term studies with BMD measurements and data on fracture incidence in women with PCOS.
Fig. 9. Serum testosterone, sex hormone-binding globulin (SHBG), free androgen index (FAI), and androstenedione levels (mean ± SEM) in control women and women with polycystic ovary syndrome in different age groups. ***p < 0.001, **p < 0.01, *p < 0.05 after BMI adjustment. SEM: standard error of the mean. Number of controls vs. PCOS at ≤30 y, 31–40 y and 41–menopause: 92 vs. 160, 54 vs. 75 and 48 vs. 63, respectively.

In Study II we evaluated the effects of metformin, one of the widely used drugs in the management of PCOS, on bone turnover in women with PCOS. Serum levels of the bone formation marker PINP and the bone resorption marker CTX were significantly decreased during the 3-month treatment with metformin in women with PCOS compared with those treated with placebo, and these changes were observed in both non-obese and obese women with PCOS (Figure 10). Studies on subjects with T2DM have shown that altered levels of bone formation and resorption markers are associated with reduced bone turnover and a slower rate of bone loss (Gilbert & Pratley, 2015). In contrast, increased bone turnover is associated with accelerated bone loss with a deterioration in bone quality and lower BMD (Srivastava et al., 2005; Szulc & Bauer, 2013). In our study, metformin treatment was associated with decreased levels of bone formation and resorption markers, leading to reduced bone turnover and slower bone remodeling, perhaps preventing bone loss.
In our study, obese women with PCOS showed decreased baseline levels of BTMs when compared with non-obese women with the syndrome. Similar results have been observed in healthy premenopausal women with higher BMI (Cohen et al., 2013; Glover, Garnero, Naylor, Rogers, & Eastell, 2008; Viljakainen et al., 2014). Furthermore, obesity is associated with increased levels of estrogens, which regulate bone metabolism via effects on osteoblasts and osteoclasts (Khosla, Oursler, & Monroe, 2012; Lash & Armstrong, 2009; Seif, Diamond, & Nickkho-Amiry, 2015). However, in our study, baseline estradiol levels were similar in both obese and non-obese women with PCOS, and the levels remained unchanged throughout the treatment period in both groups. This suggests that the decrease in BTM levels might not be related to the effect of estrogens.
The changes in hormonal and metabolic parameters seen in the metformin-treated group did not correlate with the changes in the levels of PINP and CTX. In general linear modelling, only metformin treatment showed a statistically significant interaction with changes in the levels of PINP \( (p < 0.001) \) and CTX \( (p = 0.001) \). Even though concentrations of T and values of the FAI decreased during treatment with metformin, these changes did not correlate with the changes observed in the levels of BTMs. Furthermore, the decreases in the levels of BTMs were not dependent on androgen status, as both normoandrogenic and hyperandrogenic women with PCOS showed similar declines in the levels of PINP and CTX after three months of metformin treatment (Figure 11).

![Graphs showing changes in PINP and CTX levels](image)

Fig. 11. Serum concentrations of bone formation and resorption markers (mean ± SD) at baseline and after three months of metformin/placebo treatment in normoandrogenic and hyperandrogenic (serum T > 2.3 nmol/L and/or Ferriman-Gallwey hirsutism score > 7) women with polycystic ovary syndrome. PINP: procollagen type I amino-terminal propeptide, CTX: carboxy-terminal cross-linking telopeptide of type I collagen, SD: standard deviation. *** \( p < 0.001 \), ** \( p = 0.001 \), * \( p = 0.002 \).
Body weight, covering both fat and lean mass, influences bone turnover and bone density. Increased body weight, through increased mechanical loading, stimulates bone formation (Ehrlich & Lanyon, 2002). However, in our study, metformin, but not body weight, was the influencing factor in bone turnover, as the decreases in BTM levels were seen in both obese and non-obese women with PCOS in the metformin group. Furthermore, BTMs were decreased significantly in the metformin group compared with the placebo group in women with both deficient (25OHD < 50 nmol/L) and insufficient (25OHD 52.5–72.5 nmol/L) vitamin D levels (Figure 12), suggesting that the decrease in BTM levels was not dependent on vitamin D levels.

![Figure 12](image-url)

**Fig. 12.** Serum concentrations of bone formation and resorption markers (mean ± SD) at baseline and after three months of metformin/placebo treatment in vitamin D-deficient (25OHD < 50 nmol/L) and vitamin D-insufficient (25OHD 52.5–72.5 nmol/L) women with polycystic ovary syndrome. PINP: procollagen type I amino-terminal propeptide, CTX: carboxy-terminal cross-linking telopeptide of type I collagen. SD: standard deviation. ***p < 0.001, **p = 0.001.
In conclusion, metformin treatment for three months in premenopausal women with PCOS was associated with reduced bone turnover, as reflected in decreased levels of bone formation and resorption markers, leading to slower bone remodeling, and perhaps preventing bone loss. However, the results of our study must be further validated with long-term intervention studies and fracture assessment to conclusively demonstrate the effects of metformin on bone turnover and remodeling in women with PCOS.

5.2 Retinol-binding protein 4 in PCOS (Study III)

Study III was focused, in a case-control setting, on the influence of adipose tissue dysfunction on metabolic abnormalities in women with PCOS. Retinol-binding protein 4 (RBP4) is an adipokine thought to affect systemic insulin resistance (Moraes-Vieira et al., 2014). We aimed to evaluate associations between RBP4 and various parameters in women with PCOS, and to compare RBP4 levels at different ages during reproductive life. The main result of Study III was that serum levels of RBP4 were higher in women with PCOS aged ≤ 30 years compared with controls of the same age, and this was also the case after adjustment for BMI (Figure 13). One-way ANOVA showed that serum RBP4 levels remained unchanged with age in women with PCOS ($p = 0.106$). Conflicting results have been reported as regards levels of RBP4 in women with PCOS and its role in the pathogenesis of the syndrome (Table 3). Of note, the numbers of study participants have been limited in previous studies, whereas our study included a relatively large number of subjects. In line with our results, some earlier studies have shown higher levels of RBP4 in women with PCOS (Carmina et al., 2009; Chan et al., 2010; Weiping et al., 2006).
Fig. 13. Concentrations of serum retinol-binding protein 4 (RBP4) (mean ± SD) in control women and women with polycystic ovary syndrome in different age and BMI groups. SD: standard deviation. ***p < 0.001, **p = 0.004 after adjustment for BMI and age.

In line with the results of an earlier study (Olszanecka-Glinianowicz et al., 2012), BMI-stratified analysis showed that the concentrations of RBP4 were increased in both lean and overweight women with PCOS after adjusting for age when compared with control women in the same BMI group (Figure 13). In contrast, in another study, although higher RBP4 levels were reported in obese women with PCOS compared with obese controls, similar levels were found in lean women with PCOS compared with lean controls (Hahn et al., 2007).

In women with PCOS aged ≤ 30 years, the levels of T, FAI, A, DHEAS, and triglycerides were significantly higher, and those of SHBG lower compared with controls in the same age group, after adjusting for BMI. Furthermore, RBP4 levels were positively correlated with the WHR (p = 0.020) and triglyceride levels (p = 0.001) and negatively correlated with fasting glucose levels (p = 0.002) after adjustment for BMI. Consistent with our results, RBP4 levels have previously been shown to correlate positively with WHR, but not with BMI (Weiping et al., 2006). Contrarily, some studies have reported no association between RBP4 and WHR (Diamanti-Kandarakis et al., 2008; Yildizhan et al., 2011). Conflicting findings have been reported as regards the association of RBP4 with fatty-acid metabolism and hypertriglyceridemia, with some studies reporting a strong association (Chan et al., 2010; Hahn et al., 2007; Hutchison et al., 2008) and some reporting no such association (Carmina et al., 2009; Olszanecka-Glinianowicz et al., 2012; Yildizhan et al., 2011). In our study, triglyceride levels were positively correlated with RBP4 levels in women with PCOS aged ≤ 30 years.
We found no correlations between RBP4 levels and any of the measured androgens in Study III. However, women with PCO+OA+HA and PCO+OA phenotypes had higher serum RBP4 levels when compared with the control women, after adjusting for age and BMI. Phenotypes of PCOS with HA are considered metabolically more severe compared with those without HA (Daan et al., 2014). Of note, other HA PCOS phenotypes could not be compared, as the numbers of women with these phenotypes were few. Furthermore, RBP4 levels were increased in both hyperandrogenic and normoandrogenic women with PCOS when compared with the control women (Figure 14).

In initial studies RBP4 was reported as a novel adipokine affecting systemic insulin sensitivity and glucose homeostasis (Graham et al., 2006). In PCOS, studies regarding the association between serum RBP4 levels and IR have revealed inconsistent results; some researchers have reported no association (Diamanti-Kandarakis et al., 2008; Hutchison et al., 2008) while others have reported a positive correlation between RBP4 levels and IR, but not PCOS per se (Hahn et al., 2007; Möhlig et al., 2008). We found no correlation between RBP4 levels and IR as measured by HOMA-IR. Further, women with PCOS displaying NGT had higher serum RBP4 levels when compared with controls, whereas no statistically significant differences were observed in the IFG/IGT subjects (Figure 15). These

![Fig. 14. Concentrations of serum retinol-binding protein 4 (RBP4) (mean ± SD) in control women and women with polycystic ovary syndrome stratified according to different phenotypes and androgenic status. SD: standard deviation. *** p < 0.001, * p < 0.05.](image-url)
observations suggest that the increased RBP4 levels observed in our study population are not attributable to IR per se and RBP4 might not directly affect glucose metabolism in women with PCOS.

Fig. 15. Concentrations of serum retinol-binding protein 4 (RBP4) (mean ± SD) in control women and women with polycystic ovary syndrome stratified according to glucose tolerance. NGT: normal glucose tolerance, IFG: impaired fasting glucose, IGT: impaired glucose tolerance, SD: standard deviation. ***p < 0.001.

The heterogeneity of results obtained in earlier studies of RBP4 in women with PCOS may be attributed to several factors: different cohorts studied (obese vs. non-obese; normoandrogenic vs. hyperandrogenic; normal vs. IGT), differences in PCOS selection criteria (either Rotterdam or NIH criteria), methodological differences in RBP4 assays (Western blot vs. ELISA) and differences in the methodologies used for assessing insulin resistance (OGTTs vs. clamp studies) (Hutchison et al., 2008). In conclusion, even though we found increased levels of RBP4 in women with PCOS aged ≤ 30 years, and in lean and overweight women with PCOS, we could not determine the impact of RBP4 on the metabolic derangements in PCOS. RBP4 does not seem to be a marker of IR or other metabolic derangements in women with PCOS.

5.3 Gastrointestinal permeability and dysbiosis in PCOS (Study IV)

In Study IV we aimed to evaluate intestinal permeability (IP) and dysbiosis markers in women with PCOS and their associations with hormonal and metabolic parameters. The main finding of the study was that levels of the IP markers, serum zonulin and serum FABP2, and the dysbiosis marker urinary indican were comparable in women with PCOS at age 46 compared with BMI-matched controls.
Women with PCOS had higher levels of triglycerides and FAI values compared with the control women. In subgroup analysis, normal-weight women with PCOS had decreased levels of HDL, and increased levels of LDL and triglycerides compared with normal-weight controls. Obese women with PCOS had higher FAI values compared with obese controls. Serum levels of FABP2 and zonulin, and urinary levels of indican were comparable among women with PCOS and controls in the whole study population as well as in the different BMI groups (Figure 16). BMI-stratified analysis showed that serum concentrations of zonulin increased with BMI in both women with PCOS \( (p < 0.001) \) and controls \( (p = 0.001) \), while no changes were observed in serum levels of FABP2 or urinary levels of indican.

![Fig. 16. Concentrations of urinary indican, serum fatty acid-binding protein 2 (FABP2) and serum zonulin (mean ± SD) in (A) the whole study population, and (B) control women and women with polycystic ovary syndrome stratified according to BMI. SD: standard deviation.](image)

Data on IP in women with PCOS and its role in the pathogenesis of the syndrome is very limited. Increased (D. Zhang et al., 2015) as well as similar (Cetin et al., 2019) levels of serum zonulin in women with PCOS compared with healthy controls have been reported. Of note, there were significant differences in the metabolic profiles of women with PCOS compared with the controls in the study showing elevated levels of serum zonulin, whereas in our study metabolic profiles
were comparable in the study groups. This may reflect the higher age of subjects in our study and the possibility that the groups may become metabolically closer to each other with advancing age. Serum zonulin levels were correlated with HOMA-IR values and hs-CRP levels in both women with PCOS and in controls after adjustment for BMI, suggesting that zonulin may be a marker of IR and inflammation in general.

There have been no earlier studies on FABP2, another marker of IP, in women with PCOS. Serum levels of FABP2 were similar in women with PCOS and controls, with no correlations with other metabolic parameters. FABP2 has been considered to be a marker of enterocyte injury, but not a direct marker of altered IP per se. Moreover, enterocyte damage contributes to altered intestinal barrier function (D. S. March et al., 2017). Our findings suggest that IP was not altered in women with PCOS, as reflected by the levels of gastrointestinal permeability markers. However, it has to be noted that the women with PCOS, age- and BMI-matched with controls, did not present with extreme metabolic and/or hormonal dysfunction.

Levels of urinary indican, a marker of dysbiosis, were similar in women with PCOS and controls, suggesting that dysbiosis of the gut microbiota might not be present in PCOS. This is supported by the results of a microbiome study of the same cohort, which showed no differences in the gut microbiome in women with PCOS compared with control women of late reproductive age (Lüll et al., 2020). Contrarily, other researchers have reported an association between gut microbiota and PCOS (Liu et al., 2017; Thackray, 2019). Of note, urinary indican is only a surrogate marker in the assessment of dysbiosis and may not reflect the actual status of the microbiome, even though the results of the microbiome study of the same cohort support our results. The levels of indican, FABP2 and zonulin were comparable when women with PCOS were classified on the basis of their androgenic status as normoandrogenic and hyperandrogenic. Furthermore, no significant differences were found in the levels of these markers when women were classified according to their glucose tolerance or metabolic syndrome status. In conclusion, serum levels of gastrointestinal permeability and dysbiosis markers did not differ between BMI-matched women with PCOS and controls at late reproductive age. However, serum zonulin can be considered to be a metabolic and inflammatory marker, although its concentrations did not segregate women with PCOS and controls.
5.4 Strengths and limitations

The Nordic multicenter data (Studies I and III) included a well-characterized relatively large number of subjects from a Caucasian population ranging from 18 years up to menopause, which enabled a detailed evaluation of age-related changes of the study parameters in question. The factors leading to biological variability in Studies I and II were minimized because all blood samples were collected in a fasting state. This is particularly important as regards CTX, as the levels of CTX decrease after food intake (S. Vasikaran *et al.*, 2011). The timing of samples was not scheduled according to season, but previous studies have shown no significant seasonal variation in the levels of BTMs (Blumsohn *et al.*, 2003; R. Patel *et al.*, 2001). Furthermore, the menstrual cycle phase could not be ascertained in all women, and the effect of menstrual cycle changes on BTMs could not be fully explained. However, earlier studies have shown that changes in BTM levels over the menstrual cycle are so small that the effect of the menstrual cycle can be considered to be insignificant (Delmas *et al.*, 2000; S. Vasikaran *et al.*, 2011). Moreover, the results of Study I remained the same when the samples taken in the follicular phase were analyzed separately. Also, intra-individual variations in BTM levels were not studied, as repeated measurements at different time-points were not carried out and the fracture history of the study population was not available in Study I.

A potential limiting factor in Study II could be the short duration of treatment of only three months. There is an interplay between bone resorption and formation, implying that when resorption increases formation increases, and *vice versa*. However, bone resorption is a faster process (two to three weeks) when compared with formation (three months) (Harada & Rodan, 2003; Parfitt, 1994). Thus, the study period of three months should have been sufficient to depict changes in bone formation and resorption reflected by the concentrations of BTMs. Long-term follow-up and intervention studies are necessary to conclusively demonstrate the effects of metformin on bone turnover and remodeling, as well as on BMD and fracture incidence among women with PCOS.

A limitation of Study IV is that the age of the study subjects at the time of sampling was 46 years. Of note, the metabolic profile did not differ significantly between women with PCOS and controls. The control women also had relatively high BMIs and consequently more metabolic disturbances at that age, which may narrow differences between the groups. Another limitation is that the documentation of symptoms and diagnoses of PCOS in the NFBC1966 was based
on self-reported questionnaire data (OA + H). Women with relatively mild symptoms of PCOS could be included in the study population, possibly decreasing differences between the study groups. Moreover, hirsutism scoring is easily influenced by differing perceptions of excess body hair. Ovarian ultrasonography was not available to aid the diagnosis of PCOS. However, the validity of the questionnaires in identifying women with PCOS has been documented in earlier studies on the same cohort (Karjula et al., 2017; Koivuaho et al., 2019; M. M. Ollila et al., 2016; Taponen et al., 2003; Taponen et al., 2004) (Karjula 2017, Koivuaho 2019, Ollila MM 2016, Taponen 2003, Taponen 2004).
6 Conclusions and future perspectives

The present study sought to clarify the effects of PCOS and metformin, one of the widely used drugs in the treatment of PCOS, on bone health through measurement of the circulating concentrations of bone turnover markers (BTMs). Levels of BTMs reflect underlying changes in bone mass and bone histomorphometry parameters and provide a simple assessment of bone turnover with a comprehensive view of overall bone loss. Women with PCOS aged $\leq 30$ years showed decreased bone formation, which could have a negative impact on long-term bone health in these women. However, treatment with metformin was associated with reduced bone turnover and slower bone remodeling preventing bone loss. As BTMs alone have limited predictive value as regards the incidence of fractures, which are considered to represent the primary end point of bone health, further longitudinal studies with BMD measurements and fracture-incidence data among women with PCOS are required to assess the overall skeletal health of women with PCOS.

Retinol-binding protein 4 (RBP4) is an adipocytokine affecting systemic insulin resistance and glucose tolerance in animal models. Even though we found increased levels of serum RBP4 in younger women with PCOS, the role of RBP4 in metabolic derangements in these women could not be established.

Altered intestinal permeability (IP) has been thought to play a role in the pathogenesis of various inflammatory diseases. Serum levels of zonulin, a reliable biomarker of IP, and fatty acid-binding protein 2 (FABP2), an indirect measure of increased IP, were comparable in women with PCOS and BMI-matched controls at late reproductive age, suggesting that IP does not influence the metabolic derangements in women with PCOS. However, these results should be validated in different age groups of women with PCOS to conclusively rule out the role of IP in the pathogenesis of the syndrome.
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MARKERS ASSESSING BONE AND METABOLIC HEALTH IN POLYCYSTIC OVARY SYNDROME