Dissertation

PROBIOTIC DIETARY INTERVENTION IN POLYCYSTIC OVARY SYNDROME – PREPARATION OF A RANDOMIZED CONTROLLED TRIAL

submitted by

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DECLARATION

I declare that this dissertation thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the "Guidelines of the Medical University of Graz on Good Scientific Practice"

Furthermore, I hereby declare that if artificial intelligence (AI) tools were used for the generation and/or correction of certain text passages in the creation of this work, such employment was conducted in compliance with ethical principles, academic integrity, and the regulations of my university. Additionally, it was ensured that this usage was transparently disclosed and appropriately attributed.

Graz, 18.07.2025 *Valentin Borzan, MD*

DISCLOSURES

The main publication of this thesis is referenced below:

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ABBREVIATIONS AND DEFINITIONS

17OHP	17α-hydroxyprogesterone
25OHD	25-hydroxy-vitamin D
ACE	Angiotensin-converting enzyme
ACTH	Adrenocorticotropic hormone
ADS	"Allgemeine Depressionsskala", translated: General depression scale
AE-PCOS	Androgen Excess & PCOS Society
AGE	Advanced glycation end products
AI	Aromatase inhibitors
AMH	Anti-Müllerian hormone
ARTs	Assisted reproductive technologies
ASD	Androstenedione
ASRE	American Society of Reproductive Medicine
ASV	Amplicon sequence variant
AT1	Angiotensin receptor 1
AUC	Area under the curve
BDI	Deck´s Depression Inventory
BMI	Body mass index
CAH	Congenital adrenal hyperplasia
CC	Clomiphene citrate
COCs	Combined oral contraceptive pills
CRP	C-reactive protein
DAO	Diamonioxidase
DHEA	Dehydroepiandrosterone
DHEA-S	Dehydroepiandrosterone-sulphate
ESHRE	European Society of Human Reproduction and Embryology
FAI	Free androgen index
FFG	Austrian Research Promotion Agency (“Österreichische Forschungsförderungsgesellschaft”)
FG	Fasting glucose
FSH	Follicle-stimulating hormone

fT3	Free triiodothyronine
fT4	Free thyroxine
fTesto	free testosterone
GIP	Gastric inhibitory peptide
GLP-1RA	Glucagon-like peptide 1 receptor agonists
GnRH	Gonadotropin-releasing hormone
HA	Hyperandrogenism
HbA1c	Glycated hemoglobin A1c
HDL	High-density lipoprotein
HOMA-IR	Homeostasis model assessment for insulin resistance
hs-CRP	High-sensitive C-reactive protein
I0	Fasting insulin
IC	Informed consent forms
IQR	Interquartile range
IR	Insulin resistance
KIMCL	Clinical Institute of Medical and Chemical Laboratory Diagnostics
LBP	Lipopolysaccharide-binding protein
LC/MS	Liquid chromatography tandem mass spectrometry
LDL	Low-density lipoprotein
LEfSe	Linear discriminant anaylsis effect size
LH	Luteinizing hormone
LOD	Laparoscopic ovarian drilling
LOS	Laparoscopic ovarian surgery
Lp(a)	Lipoprotein (a)
LPS	Lipopolysaccharide
MAFLD	Metabolic dysfunction-associated steatotic liver disease
MDA	Malondialdehyde
MetS	Metabolic Syndrome
mFG	Modified Ferriman-Gallwey score
NAFLD	Non-alcoholic fatty liver disease
NIH	National Institute of Health
oGTT	Oral glucose tolerance test
OM	Oligo-/Amenorrhea

OR	Odds ratio
OS	Oxidative stress
OTU	Operational taxonomic unit
PCoA	Principal coordinate analysis
PCOM	Polycystic ovarian morphology
PCOS	Polycystic Ovary Syndrome
PD	Phylogenetic diversity
PTH	Parathyroid hormone
PYY	Peptide YY
RCT	Randomized controlled trial
RDA	Redundancy analysis
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
sCD14	Soluble Cluster of Differentiation 14
SHBG	Sex-hormone-binding protein
SSRI	Selective serotonin reuptake inhibitors
T	Testosterone
TAC	Total antioxidant capacity
TSH	Thyroid-stimulating hormone
TT	Total testosterone

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ABSTRACT IN GERMAN

Weltweit ist jede fünfte Frau vom Polyzystischen Ovar-Syndrom (PCOS) betroffen, bei dem eine vermehrte Androgenproduktion zu unregelmäßigen Zyklen, unerfülltem Kinderwunsch und Hirsutismus führen kann. Zusätzlich führt PCOS häufig zu einer Insulinresistenz mit Gewichtszunahme, einem Typ 2 Diabetes, einem erhöhten kardiovaskulären Risiko sowie einem hohen Leidendruck unter den Betroffenen.
In den letzten Jahren kristallisierten sich Probiotika als potenzielle neue Behandlungsmethode für PCOS, indem sie die Androgenwerte senken und/oder die metabolischen Folgen lindern können. Jedoch mangelt es noch an robusten klinischen Daten zu dieser Fragestellung.
Diese Dissertation widmet sich dem Design einer randomisiert kontrollierten Studie (RCT), die ein Probiotikum mit einem Placebo und dem PCOS-Standarttherapeutikum Metformin in Frauen mit PCOS vergleicht.
Zu diesem Zweck wurde eine Pilotstudie durchgeführt, die drei kommerziell verfügbare Probiotika der Firma Institut Allergosan, Graz, Österreich miteinander verglich, um das geeignetste Probiotikum für die RCT zu finden und die Methodik der RCT-Studie zu testen.
Zusätzlich wurde eine retrospektive Analyse einer vorhandenen PCOS-Kohorte durchgeführt, um die metabolischen Folgen abhängig von den klinischen Beschwerden zu untersuchen, und um den primären Endpunkt der RCT-Studie zu definieren, da von diesem auch die Fallzahlberechnung abhängt. Basierend auf den Erkenntnissen dieser beiden Studien wurde das Design und der Aufbau der großen RCT-Studie entwickelt.
Die Kohortenstudie konnte zeigen, dass Frauen mit erhöhtem freien Testosteron das höchste Risiko für die Entwicklung einer Insulinresistenz hatten, daher wurde dieser Parameter als primärer Endpunkt für die RCT-Studie herangezogen. Da in der Pilotstudie OMNi-BiOTiC® metabolic die größte mediane Reduktion von freiem Testosteron zeigen konnte, wurde das Probiotikum den anderen beiden bevorzugt.
Basierend auf diesen Vordaten konnte die RCT-Studie in Anlehnung an die Pilotstudie viele Methoden übernehmen, wobei Lektionen aus beiden Vorläuferstudien in das Design einflossen.
Bei einem erfolgreichen Abschluss der RCT-Studie könnte sich ein neuer therapeutischer Zweig für Frauen mit PCOS auftun.

ABSTRACT IN ENGLISH

Polycystic Ovary Syndrome (PCOS) affects up to 20% of all women worldwide, leading to irregular menstrual cycles, fertility issues and hirsutism caused by elevated androgen levels. Further metabolic conditions and complications include insulin resistance and weight gain, type 2 diabetes, an increased risk of cardiovascular diseases and a potentially severe reduction in their quality of life.

Probiotics have been investigated in PCOS as a new treatment approach, potentially reducing clinical symptoms and improving their metabolic health. However, reliable data on this topic are still scarce.

This thesis summarizes the process of designing a large randomized-controlled trial (RCT), comparing probiotics with placebo and the standard treatment for PCOS, metformin, to determine their potential in reducing symptom severity.

For this purpose, a pilot trial was set up in order to test the proposed methodology and select the most promising probiotic out of three distinct commercially available probiotics from Institut Allergosan, Graz, Austria. In addition, a retrospective analysis of a well-established PCOS cohort was conducted to determine the impact of each particular PCOS symptom on the metabolic health in women with PCOS, which would later be used to determine the primary endpoint of the large-scale RCT as well as the sample size calculation needed for later recruitment. The study design, including study visits, recruitment process, endpoint selection and choice of probiotic intervention, was determined based on the methods and results of these two preparatory studies.

In the cohort study, women with elevated free testosterone levels were at most risk of developing insulin resistance and other metabolic sequelae, which is why free testosterone was selected as the primary endpoint measure for the RCT. Based on median free testosterone changes in the pilot trial, OMNi-BiOTiC® metabolic was selected as the probiotic of choice for the RCT.

Based on the pilot trial methodologies, the RCT was then designed using additional findings from both trials. If proven successful, probiotics might become a new treatment option for women with PCOS.

1 Introduction

1.1 Definition and symptoms

Polycystic Ovary Syndrome (PCOS) is an endocrine and metabolic disorder affecting 6 to 22 percent of all women worldwide. The discrepancy in reported incidence rates can mostly be attributed to the varying definitions used in the reporting studies (1,2).

The most common symptoms include excess androgen production, oligo- or anovulation and –oligo-/amenorrhea (OM), infertility, alopecia, hirsutism, insulin resistance (IR) with progression to type 2 diabetes and obesity as well as other metabolic and hormonal changes. PCOS can feature any number of those symptoms, making it sometimes difficult for clinicians to correctly recognize and assign individual symptoms.

In the past, there have been several definitions of PCOS. In 1990, the National Institute of Health (NIH) published the first diagnostic criteria, establishing PCOS as a combination of hyperandrogenism (HA) and oligo-/anovulation.

These criteria were expanded upon at a PCOS conference in Rotterdam in 2003, establishing the "Rotterdam criteria". Hyperandrogenism was copied from the NIH criteria, while oligo-/anovulation was changed to ovarian dysfunction, thereby including oligo-/amenorrhea as well. The third criterion established was the sonographic presence of polycystic ovarian morphology (PCOM). The presence of at least two Rotterdam criteria was required for a PCOS diagnosis (1).

While other diagnostic criteria have been proposed since, in 2012 the NIH also recommended using the Rotterdam criteria, while also emphasizing the importance of differentiating between the four PCOS phenotypes resulting from the possible combinations of present criteria (1).

In order to highlight this, a brief overview and understanding of PCOS pathophysiology is following in the upcoming part of the thesis in view of physiological and pathophysiological processes of the hormonal and metabolic regulation of PCOS.

1.1.1 PCOS phenotypes – a comparison and beyond

Based on the Rotterdam criteria (HA, OM, PCOM), there are four distinct PCOS phenotypes: phenotype A contains all three criteria, B consists of HA and OM, HA and PCOM make up phenotype C, while phenotype D does not show any sign of HA, only PCOM and OM.

Notably, IR, obesity and metabolic risk profiles do not play a role in PCOS diagnosis, despite having a profound impact on its pathophysiology and needing consideration regarding the possible treatment options.
In addition, the current PCOS definition based on at least two out of three criteria excludes any woman with only one criterion, including those with HA. However, as described in chapter “1.2 Pathophysiology”, HA is the main driving factor behind PCOS pathophysiological pathways.
Several studies have previously separated PCOS phenotypes and assessed their heterogeneous makeup and symptom expression as well as metabolic biomarkers (1,3,4). However, women with only one Rotterdam criterion have not been included in the comparison, as they do not fit the definition of PCOS, despite having some of the same symptoms, and an increased risk for later sequelae.

1.2 Pathophysiology

1.2.1 Physiological menstrual cycle

Under physiological conditions, follicle stimulating hormone (FSH) is released in the first half of the menstrual cycle in order to stimulate follicular development of a primordial follicle into a tertiary follicle ready for ovulation. The latter follicle contains the secondary oocyte surrounded by granulosa cells converting androgens to estrogens while stimulated by FSH and theca cells. The theca cells produce androgens while stimulated by luteinizing hormone (LH) during the second half of the menstrual cycle after the conversion of the follicle into the corpus luteum. LH also stimulates the production of progesterone in the corpus luteum to facilitate endometrial buildup for a possible implantation of the blastocyst in the case of a pregnancy. At the end of the cycle, progesterone levels drop, which leads to the shedding of the endometrium, causing menstrual bleeding and starting the cycle anew (5,6).
LH and FSH levels are both regulated by the gonadotropin releasing hormone (GnRH), which is released in a pulsatile way. GnRH itself is negatively regulated by the heightened progesterone levels during the latter half of the menstrual cycle (6).

1.2.2 Androgen production in women

Androgens are produced in equal amounts by the ovaries (via LH stimulation) and the adrenal glands via adrenocorticotropic hormone (ACTH) stimulation. The two main circulating androgens are androstenedione (ASD) and testosterone (T). Androstenedione is produced from Dehydroepiandrosterone (DHEA) and can itself be converted to testosterone in peripheral tissues. Many androgens are converted to estrogens in the granulosa cells or again in peripheral tissues. However, testosterone itself also has many important functions in women. Among others, it serves as an anabolic hormone for muscle and bone tissues, it stimulates erythrocyte production via erythropoietin and increases the libido (7).

It´s noteworthy that there is no negative feedback for androgen production, the more the trophic hormones LH and ACTH stimulate their respective organs, the more androgens are produced (7). 60 to 70 percent of circulating T is bound by both the sex-hormone-binding protein (SHBG) and approximately 30 percent to albumin. Only 2 to 4 percent of total circulating T molecules exist in an active, free form (free Testosterone, fTesto). SHBG-bound testosterone has to pass the cell membrane via special transport proteins. In contrast, fTesto and - to a lesser degree - albumin bound testosterone pass cell membranes freely and can therefore activate androgen receptors located within (8).

1.2.3 PCOS: How it all starts

A central component of PCOS pathophysiology is the dysregulation of androgen production, leading to HA. While the exact processes remain largely unknown, a combination of genetic, epigenetic and environmental factors are thought to play a role in shifting androgen metabolic pathways towards HA. While genetics are considered to contribute approximately 10 % to PCOS incidence, fetal exposure to androgens, Anti-Müllerian-hormone (AMH) and/or other nutritional or environmental factors might initiate epigenetic changes, which may be activated in later life by certain lifestyle choices (9,10).

Importantly, several conditions in women with PCOS lead to HA. First, the theca cells in women with PCOS are enlarged, increasing the disposition to androgen production (11). One common mechanism driving this change is excess insulin secretion due to insulin resistance (IR, see below) (12). Second, androgen secretion decreases the expression of progesterone receptors in the hypothalamus, lowering GnRH-sensitivity to LH levels and impairing the negative feedback usually regulating GnRH release schedules. Third, the pulsatile release GnRH is affected, altering the LH/FSH ratio and disrupting the proper concentration levels during the menstrual cycle (13).

In turn, this shift in the pituitary gland towards LH secretion instead of FSH secretion leads to several changes. On the one hand, LH overstimulates the overproducing theca cells further, increasing androgen levels. On the other hand, FSH insufficiency leads to the cessation of follicle development and in turn to oligo- or anovulation, which again leads to OM due to the missing LH depletion stimulus for the endometrium. In addition, the conversion from androgens to estrogens in the granulosa cells is halted, as the necessary enzyme aromatase is FSH-dependent (13).
These processes taken together lead to a self-perpetuating positive feedback loop of excess androgen production. It is the first of several vicious metabolic and hormonal cycles perpetuating PCOS.

1.2.4 From hyperandrogenism to insulin resistance, and back again

The hyperandrogenic dysregulation in women with PCOS also has a dramatic impact on the glucose and lipid metabolism via several different pathways, and vice versa.
At the center of the metabolic changes is insulin sensitivity in peripheral tissues as well as the central nervous system. The subcutaneous and the visceral fatty tissue as well as skeletal muscle tissue have androgen receptors, which normally play a role in insulin receptor sensitivity to insulin. In a hyperandrogenemic state, the insulin sensitivity of the receptors is lowered, leading to decreased lipolysis and increased fatty tissue hypertrophy, resulting in an increased risk of obesity in women with PCOS (13). The β-cells in the pancreas in turn secrete more insulin to compensate for this issue, which results in a state of hyperinsulinemic IR. IR in turn can lead to gestational diabetes or type 2 diabetes, irrespective of the presence of obesity, as many women with PCOS who are not overweight also have IR or diabetes (13).
The brain also plays a role in insulin sensitivity regulation. Insulin can pass the blood-brain barrier, and increased brain concentrations of insulin have led to decreased appetite and weight loss, though most studies conducted on the topic could only demonstrate this effect in men. A recent study by Hummel et al. has shown that increased brain insulin levels have the same effect in women as in men, however only during the follicular phase of the menstrual cycle. In the luteal phase of the cycle, brain insulin did not show any decrease in appetite or weight (14). Since women with PCOS have irregular menstrual cycles with an overexpression of LH over FSH, peripheral IR is further enhanced.
IR itself also feeds back to increasing HA in several ways. First, it stimulates GnRH secretion in the hypothalamus, altering its pulsatile release cycle and thereby promoting LH secretion over

FSH secretion. Second, insulin increases ACTH sensitivity in the adrenal glands, leading to excess androgen production. Third, the pituitary gland also has insulin receptors, which lead to increased LH and FSH secretion. Last but not least, the ovaries have insulin receptors as well, which, upon activation, stimulate further androgen secretion via proliferation of theca cells. However, unlike the receptors in fatty or muscle tissues, ovarian insulin receptor sensitivity to insulin remains unchanged during states of IR (12,13).

These metabolic changes create another vicious cycle, reinforcing hyperandrogenic conditions and promoting PCOS symptoms. Interestingly, IR occurs almost exclusively in PCOS women who have a phenotype exhibiting HA, whereas HA can occur without the presence of IR (3).

1.2.5 Anti-Müllerian-hormone: Another vicious cycle

While the interactions between androgen and insulin pathways play a major role in PCOS symptom expression, other metabolic processes are also involved.

AMH is a major player in PCOS pathogenesis. Under physiological conditions, AMH is expressed in granulosa cells of the early follicles and inhibits their development as an antagonist to FSH in order to prevent premature follicular depletion and promote selective FSH-dependent follicle selection. It regulates aromatase expression as well as FSH sensitivity of the granulosa cells. AMH is primarily expressed in the preantral and small antral follicles for this purpose, decreasing its expression in later follicle stages (10).

In PCOS however, AMH is expressed in higher concentrations, leading to an overinhibition of FSH as well as aromatase expression in the granulosa cells. This in turn leads to the arrest of follicular development, resulting in many small antral follicles accumulating in the ovaries. As these follicles have a high AMH expression, this creates another vicious cycle of HA, though not present in all phenotypes of women with PCOS (10).

1.2.6 Oxidative stress and low grade inflammation

Oxidants are another biochemical component of PCOS pathophysiology. Physiologically, reactive oxygen and nitrogen species (ROS, RNS respectively) regulate various facets of cellular metabolism, ensuring their proper function. When produced in excess however, they react with cellular structures and molecules in an uncontrolled manner, disrupting their function and

potentially causing cell, tissue and organ damage. This state is referred to as oxidative stress (OS) (15).

ROS play a vital role in the selection and development of the dominant follicle during the follicular phase by preventing follicular growth. Antioxidants ensure the progression of the dominant follicle by preventing the ROS from interacting with it (15).

In PCOS, antioxidant concentrations are decreased, promoting follicular development arrest, while also increasing OS byproducts in the ovaries like advanced glycation end products (AGEs). OS also seems to be a major driver of insulin resistance in peripheral tissues by disrupting normal insulin receptor function, thereby creating one of the main vicious cycles of PCOS pathophysiology. In addition, OS is an important factor in the development of cardiovascular disease as well as numerous cancers. In PCOS, several studies have found an increased risk of developing endometrial and ovarian cancer, with OS providing a possible explanation (15).

OS is a vital component of inflammatory processes, which are also promoted by the metabolic changes in PCOS, especially IR and obesity. Women with PCOS exhibit higher inflammatory marker concentrations than healthy controls, promoting low grade inflammation, which also plays a role in PCOS pathogenesis. However, it is unclear if low grade inflammation is a result of PCOS directly, or a consequence of secondary metabolic changes, such as IR and obesity (16).

1.3 Treatment options

As PCOS is such a multifaceted disease with a complex pathophysiology affecting many different organ systems and metabolic pathways, there is a priori no treatment option that can address every possible symptom combination in an individual patient. Instead, there are many different treatment options, most of which treat specific symptoms. A summary of the most common options is supplied in table 1.

Treatment Options for PCOS symptoms						
Treatment	**PCOS Symptoms**					**Comment**
	Hirsutism, acne, allopecia	Oligo-/Amenorrhea	Infertility	Insulin resistance	Obesity	
Lifestyle intervention	+	+	+	++	++	Good results when successful, difficult to maintain, goal-setting is important
Combined oral contraceptives	++	++	-	-	-	Very effective in treating clinical symptoms of HA, unsuitable for women with active child-wish, have several side effects and risks associated
Metformin	-	+	+	++	(+)	Unique mechanism, combinable with other treatments, suitable for women with child wish, common gastrointestinal side effects
Anti-androgens	++	(+)	-	-	-	Effective in treating skin conditions associated with HA, common and severe side effects possible, thereby limited use in practice
Aromatase-inhibitors	-	++	++	-	-	First-line therapy in ovulation induction
Clomiphene-citrate	-	+	+	-	-	First-line therapy in ovulation induction, though lower response rate than aromatase inhibitors
Gonadotropins	-	++	++	-	-	High success rate, high rate of multiple pregnancies, second-line ovulation induction therapy due to costs and logistical problems
Laparoscopic ovarian surgery/drilling	-	+	+	-	-	Third-line option for ovulation induction, cost-intensive and high risk of complications
Bariatric surgery	(+)	(+)	(+)	++	++	For morbidly obese PCOS patients, high risk of postinterventional nutritional deficits, increased risk of pregnancy complications, third-line therapy
In-vitro-fertilization	-	-	+	-	-	Third-line therapy for infertility, high costs, low rate of successful live births

Inositol	-	+	+	+	(+)	Intracellular second messenger, method of action not fully known, low degree of evidence
Glucagon-like peptide 1 receptor agonists	+	+	(+)	+	++	Antidiabetic and anti-obesity medication, off-label-use in PCOS, promising results, need more research, most effective in overweight women with PCOS, high rate of side-effects

Table 1: Therapeutic options for PCOS symptoms. ++: very effective; +: effective; (+): partially effective -: not effective; The table was translated and cited from (17), with the addition of GLP-1RA, which were not present in the original publication;

1.3.1 Lifestyle intervention

Multiple studies have shown the beneficial effects of lifestyle intervention on reducing the adverse effects of IR and obesity as well as to a lesser extent hirsutism and other effects of HA. A combination of dietary changes and physical activity intervention is recommended in order to reduce androgen levels and improve quality of life in women with PCOS, especially in obese women with PCOS (18).

Nutritional education and dietary changes should especially be considered a priority among adolescents showing early signs of PCOS, with a special focus towards reducing the glycemic index and increasing fiber and complex carbohydrate intake. Depending of the body weight and composition, hypocaloric diet might also be considered (19).

A commonly recommended diet consists of low carbohydrate ketogenic diet, whereby carbohydrate intake is reduced significantly and exchanged with an increased fat intake. Recently, ketogenic diet has been show to more effectively reduce body weight, waist-to-hip ratio as well as improve reproductive and metabolic parameters in obese women with PCOS compared to a traditional Mediterranean diet. The latter is also often recommended due to consisting of high amounts of plant fibers and unsaturated fatty acids (19,20).

In addition to nutritional lifestyle changes, regular exercise is also recommended for improving the fertility and metabolism of women with PCOS. There are several approaches to this: intermediate intensity aerobic training with a recommended training time of at least 150 minutes per week as well as high intensity training of at least 90 minutes per week, possibly incorporated into the aerobic exercise sessions (21,22).

However, lifestyle changes can often be very challenging to maintain. During the recruitment for PCOS studies at the endocrinology outpatient clinics at the Medical University of Graz, many

overweight women with PCOS anecdotally reported no weight loss despite rigorous training sessions and dietary interventions.
Besides lifestyle intervention, pharmacological therapy is also an important component of PCOS treatment regimens.

1.3.2 Medication altering androgen metabolism

Combined oral contraceptive pills (COCs) are very commonly used to treat symptoms of HA in women with PCOS, in particular hirsutism, alopecia and OM. They are also often combined with other treatments to more broadly address the individual needs of the patient. However, COCs cannot be used by women who have an unfulfilled child wish. In addition, they can increase IR and triglyceride levels, and possible side effects include occurrence of thromboembolic events, weight gain and migraines, among others (23,24).
Anti-androgenic medications (finasteride, flutamide, spironolactone) are also in use, usually in combination with other treatments, mainly to treat skin symptoms of PCOS like hirsutism, alopecia and acne. However, even more than COCs, their side effects can be very significant and in addition, patients need to use contraceptive methods during sexual intercourse due to their teratogenic potential in male fetuses (23).

1.3.3 Ovulation induction medication

In contrast, clomiphene citrate (CC) as an estrogen receptor modulator was the first line therapy for ovulation induction in women with PCOS for a long time, before being replaced by aromatase inhibitors (AI), such as letrozole. Ironically, by blocking aromatase-based conversion of androgens to estrogens further in PCOS, the lack of estrogen stimulates the secretion of FSH in the pituitary, increasing the likelihood of a dominant follicle being selected and developed. Letrozole has been shown to be superior to CC in inducing ovulation, and despite early concerns of teratogenic potential in male fetuses, subsequent studies have not been able to show such a potential. Both medications are, however, not always successful in inducing ovulation, and CC in particular has some anti-estrogenic effects, sometimes preventing pregnancies despite enabling ovulation (23). The most successful hormonal infertility treatment method consists of regular FSH injections in order to directly induce follicle development. While this method has been shown to be more effective than a combination of CC and metformin when comparing pregnancy rates in an RCT,

the injections are prohibitively expensive for many potential patients, and in additions there is an increased incidence of multiple pregnancies, which have a higher risk of pregnancy complications (25,26).

1.3.4 Metformin

Apart from medications affecting sex hormone metabolism, the insulin sensitizer metformin has been used for PCOS treatment for decades and is in many ways unique compared to other pharmaceutical options.

A chemical derivative of goat´s rue (galega officinalis), metformin or dimethylbiguanide was discovered at the beginning of the twentieth century as a means to lower blood sugar levels in diabetes patients. However, for a long time it was outshone by other biguanides due to their higher potency. Despite being discovered in 1918, it was only established as the first-line therapy for type 2 diabetes in the 1990s (27). Since then, metformin has quickly become the most prescribed medication for type 2 diabetes worldwide (28).

Metformin inhibits gluconeogenesis in the liver, reducing fasting glucose levels, as well as improving peripheral tissue sensitivity to insulin. It also stimulates intracellular adenosine monophosphate kinase activity, leading to increased insulin dependent glucose transporter 4 expression in cell membranes and therefore decreased fasting glucose levels and improved peripheral insulin sensitivity (28–30).

In PCOS, metformin disrupts one of the main vicious cycles of generating HA by improving insulin sensitivity and lowering IR in peripheral tissues. By this process, metformin improves symptoms and serum levels of HA as well as OM.

1.3.5 Inositol

Inositol is classified as a dietary supplement and acts as a second messenger involved in many metabolic cellular pathways, including insulin metabolism. Some studies have found improved menstrual cycle regularity and ovulation rate in treated women with PCOS (31,32). However, there are many different dosages on the market and more studies are needed to determine the optimal treatment plan. In addition, a commonly used isomer of inositol, D-chiro-inositol, has been found to act as an aromatase inhibitor and may increase androgen levels in women with PCOS.

1.3.6 Glucagon-like peptide 1 receptor-agonists

Glucagon-like peptide 1 receptor agonists (GLP-1RA) have been used for more than a decade as antidiabetic medication (33).

GLP-1 is a peptide hormone secreted in the small intestine and stimulates insulin secretion in times of hyperglycemia. GLP-1RA have an extended half-life compared to GLP-1, enabling their use as treatment options for type 2 diabetes and obesity. In addition to their insulin-secreting and β-cell-proliferating effect, they reduce appetite via stimulation of the central nervous system. They also inhibit glucagon release, which in turn leads to decreased glucose levels in times of hunger by reducing the breakdown of glycogen in the liver (which is glucagon-dependent), and they prolong gastric emptying (34). All of these effects lead to decreased glucose levels, increased satiety and lower appetite.

GLP-1RA have experienced a big hype in recent years due to their weight-losing effects in addition to their antidiabetic role. Naturally, this makes them interesting treatment options for PCOS patients, who often struggle both with IR and obesity.

A review by Cena et al. in 2020 assessed several small studies using GLP-1RA as treatment options for PCOS and found significant weight loss and reduction of androgen levels in most studies, while finding mixed results with regards to IR and menstrual symptom reduction (35).

A more recent RCT comparing liraglutide to placebo in 82 obese women with PCOS found significantly higher weight loss and free androgen index reduction in women treated with liraglutide, however 60 % of liraglutide patients experienced mild to moderate gastrointestinal symptoms, compared to 18 % of placebo patients (36).

Another RCT by Xing et al. compared metformin to metformin and liraglutide in 60 overweight women with PCOS for a 12 week intervention period. The combined therapy group showed a significantly higher improvement regarding androgen levels, while body weight, metabolic markers as well as menstrual symptoms improved in both groups, showing no significant differences between the two interventions. In both intervention groups, mild gastrointestinal symptoms were reported in the initial two weeks of intervention, with most resolving spontaneously after two weeks. Exact numbers were not reported, though the authors emphasize that no drop-outs occurred due to any side effects (37).

In summary, while GLP-1RA provide an interesting novel approach to target metabolic comorbidities in PCOS, more research is needed to establish them as routine treatment options in PCOS. Specific limitations seem to be their effectiveness on mostly overweight or obese women

with PCOS, as well as their side effects, which seem to occur even more commonly than in metformin.

1.3.7 Assisted reproductive technology

In patients with oligo-/anovulation non-responsive to first- and second-line lifestyle and pharmacological treatments, assisted reproductive technologies (ARTs) such as in-vitro-fertilization or intracytoplasmic sperm injection may provide an additional option to induce ovulation. However, the prohibitive costs associated with the procedures, the limited rate of live births as well as the increased risk of ovarian hyperstimulation syndrome in PCOS severely limit the usefulness of ARTs and they are considered third-line treatment options (26).

1.3.8 Surgical intervention

While surgical treatment of PCOS might not sound intuitive at first, there are two main areas of surgical intervention that have been considered in PCOS treatment plans.

Laparoscopic ovarian surgery/drilling (LOS/LOD) is used for removing a segment of ovarian tissue or drilling holes into the ovary and partially destroying the surrounding tissue in order to stimulate follicular development in the remaining ovarian tissue (38). This process works by decreasing the amount of androgen and AMH-producing preantral and small antral follicles, thereby reducing the inhibiting effects on the follicular development of the remaining follicles. For both surgical options, LOS and LOD have been shown to equal CC and other monotherapies in outcomes regarding ovulation induction and pregnancy rate. However, CC plus metformin have been shown to be superior in combination together and both interventions carry typical risks of surgical options, including risk of infection, adhesional buildup as well as an increased rate of complications during and after the procedures for overweight and obese women, a very common patient group in PCOS (38).

The second area of interest in PCOS concerns bariatric surgery in morbidly obese women with PCOS. Weight reduction has been connected to improved glucose and insulin metabolism, thereby reducing PCOS comorbidities. In addition, obesity is an additional risk factor for pregnancy complications. However it should be noted that evidence of bariatric surgery as a treatment option for infertility and PCOS in particular is extremely limited. One study conducted in China with 90 obese PCOS participants compared bariatric surgery to metformin plus COCs and found the

surgical option to be significantly more effective in reducing weight and inducing ovulation. Furthermore, BMI reduction was shown to be the primary driving factor behind ovulation onset, thereby solidifying weight loss and management as a vital factor in PCOS treatment regimens (39). A meta-analysis published a year before found a significant improvement of androgen and IR parameters in patients with PCOS after bariatric surgery (40).

It should be noted however, that this option is only available to morbidly obese women with PCOS and should only be considered after first-line lifestyle and pharmacological interventions fail. In addition, pregnancies should be avoided in the first 12 months post-surgery in order to prevent pregnancy complications, and patients should be monitored closely during and after the weight loss in order to minimize further risks, such as malabsorption and vitamin and other deficiencies that might have to be substituted throughout life.

1.3.9 Future aspects

In summary, while there are many different treatment options, they all treat specific and different symptoms of PCOS and all have various side effects or caveats limiting their use.

Therefore, there is a large interest to discover and/or develop new treatment approaches for women with PCOS, who are not eligible for current treatments or who do not respond to current treatment regimens adequately.

However, before a clinical trial for a new approach can be set up, it is of vital importance to determine potential new treatment approaches as well as the outcome parameters for such a trial determining success or failure, especially in such a heterogeneous endocrine and metabolic disorder as PCOS. In addition, understanding the different phenotype expressions and their impact on metabolic health improves the determination on who might benefit most from different treatment approaches.

1.4 Probiotic intervention – A new and exciting approach?

1.4.1 Microbiome

The human microbiome sums up the entirety of all microbiotic organisms inhabiting the human body in a symbiotic relationship. For metabolic health purposes, the gut microbiome in particular is of vital importance. It consists of bacteria, viruses, archaea and eucaryotes, and it is more

variable and diverse than our own genome by several orders of magnitude (41). Even identical twins can have vastly different gut microbiome compositions (42).

The gut microbiome plays an important role in several metabolic and immunological processes (41), and in order to understand these, here is a brief overview of our current knowledge on the topic.

The microbiome itself can be divided into bacteria, viruses, fungi and archaea. Our first major contact with microbiota occurs during birth. Depending on the mode of delivery, either vaginal or skin microbiota dominate the first colonization process of the gut (43), while the gut microbiome composition and diversity changes drastically over time, as babies develop and adapt in their nutrition. At two-and-a-half years, the gut microbiome exhibits many characteristics of the adult microbiome composition (44).

In most cases, our microbiome remains stable and imparts a healthy metabolic and immunological function, however there are many factors that can further influence the microbiome composition, such as infections, use of antibiotics and a multitude of chronic diseases, including obesity, type 2 diabetes, chronic gut inflammation and even degenerative neurological diseases (41,45,46). Many of these chronic illnesses are especially associated with decreased microbiome diversity and increased gut inflammation and permeability.

Therefore, modulating the gut microbiome in order to improve disease symptoms became a great interest of many researchers studying these topics. One major way of modulating the gut microbiome is via probiotic supplementation.

1.4.2 Probiotics

Probiotics are defined by the WHO as live bacteria that confer a beneficial effect on the host after consumption in the right amount. They are not to be confused with prebiotics, which are food components that confer a health benefit upon consumption via gut microbiota metabolic processes. The term symbiotic is used when a probiotic and a prebiotic are used simultaneously in a product (47).

Many foods already contain probiotics, either through industrial manufacturing (such as fermented foods in order to change texture or flavor) or via their very nature such as yoghurt, cheese or wine. In most cases, probiotics stem from the lactic acid producing group of bacteria, as they are easily cultured, controlled and are abundant in the human gut (48).

In general, probiotic use is considered safe, however immunocompromised, intensive-care, critically-ill infants and postoperative or hospitalized patients may be at higher risk of developing potentially serious adverse effects of probiotic use, such as sepsis, fungal infections or gut ischemia (49).

In recent years, gut microbiome health has been linked to numerous diseases from the entire medical spectrum, either through a reduction in diversity or via changes in microbial composition, or both. As a result it stands to reason that there have been numerous attempts to improve symptoms and quality of life in affected patients through probiotic intervention, aka introducing healthy bacteria into a dysbalanced environment.

With close to 50.000 publications in the MEDLine database (49.729 search results when searching for “probiotic”, accessed via: https://pubmed.ncbi.nlm.nih.gov/?term=probiotics on July 15, 2024), among them more than 12.000 review articles (12.289 search results when using the above search with the additional filter “Article type: Review”, accessed via: https://pubmed.ncbi.nlm.nih.gov/?term=probiotics&filter=pubt.review on July 15, 2024) as of July 15, 2024, the amount of available literature is simply staggering, and the number of publications continues to grow each year (based on the above mentioned searches and the number of search results sorted by year).

This is mostly due to the broad application spectrum of probiotics, as there are many areas of interest with regards to microbiota modulation and subsequent health improvements in a wide number of diseases and situations.

Probiotics in pregnancy and infancy

As the first few years can be considered formative for the later microbiome composition, the initial colonization of the gut during the birth process and in infancy is of particular interest. Babies born via caesarean section show less diversity in their microbiome and also altered composition with regards to potentially harmful microbiota, such as *Escherichia coli* when compared to vaginally-delivered newborns. A systematic review by Martín-Peláez et al. demonstrated that prebiotics, probiotics and synbiotics can mitigate the gap in microbiome composition between the two birth method cohorts, thus reducing the risk of metabolic sequelae. Even more remarkable, probiotic use during the last stages of pregnancy and lactation period can improve the microbiome composition of breast milk, increasing the amount of beneficial bacteria in breast-fed newborns, independently of the birthing method (50).

In prematurely born infants, probiotic supplementation has been shown to reduce overall mortality as well as incidence of necrotizing enterocolitis, with a very low incidence of probiotic-induced sepsis as a serious complication (51,52).

Probiotics in gastrointestinal disorders

Due to the oral administration of probiotics and their colonization of the gastrointestinal tract, probiotic intervention is also of particular interest for patients suffering from a number of gastrointestinal disorders.

Probiotics help mitigate the symptoms of antibiotic-associated diarrhea as well as clostridium difficile associated diarrhea (53,54). In contrast, a recent Cochrane systematic review concluded that infectious diarrhea without presence of clostridium difficile showed no or little improvement upon probiotic administration (55).

There is also some evidence that probiotics may improve outcomes of ulcerative colitis when used concurrently with 5-aminosalicylates, although no such benefits have been found in patients with Crohn´s disease (56,57).

Irritable bowel syndrome is another common disorder for which probiotics have been investigated as a treatment option. However, despite tenuous results in some studies that patients suffering from the condition may benefit most from multistrain probiotics used over 8 weeks or more, evidence on the matter is very limited (58,59).

Probiotics in metabolic disorders

Probiotics have been found to reduce weight in overweight or obese subjects, either as a monotherapy or as part of a broader lifestyle modification therapy (60), improve glucose metabolism and inflammation markers in adults with type 2 diabetes mellitus (61,62), lipid profile parameters to varying degrees (63), as well as modulate potential sequelae of the conditions mentioned above.

Positive effects of probiotics have been found in patients suffering from non-alcoholic fatty liver disease and non-alcoholic steatohepatitis (64,65) as well as chronic kidney disease (66).

Probiotics for immune system modulation

Due to their connection to and importance for immune response modulation, probiotics have been investigated extensively for the improvement of immune system functions, both locally and systemically.

Locally, probiotics improve gut barrier function, reducing the amount of toxins and other potentially harmful substances from entering the blood stream from the gut lumen, thereby reducing low-grade systemic inflammation (67).

As gut microbiota play an important role in the formation and functioning of the gut- and mucosa-associated lymphoid tissues, as well as maintaining the equilibrium of pro- and anti-inflammatory factors within the gut, introducing healthy bacteria to restore gut dysbiosis was investigated as a possible supportive therapeutic option for various autoimmune diseases, with both direct and indirect connections to the gastrointestinal tract (68).

Specific probiotic strains have been shown to exhibit positive effects in systemic lupus erythematosus, rheumatoid arthritis, type 1 diabetes mellitus and psoriasis, as well as several other types of autoimmune arthritis, though data is limited in most cases and sometimes contradictory (68–70).

Probiotics for neurological and psychological disorders

It is well established that the gut microbiome is directly connected to the central nervous system via the gut-brain axis. Many neurodegenerative and neuropsychological diseases such as Alzheimer´s dementia, Parkinson´s disease and depression are associated with gut dysbiosis (71). Therefore the introduction of probiotics to improve microbiome composition and diversity in the gut is of particular interest in these disorders.

In Alzheimer´s dementia, probiotic supplements containing *Bifidobacterium* and *Lactobacillus* have been shown to improve cognitive functions and memory retention after as short as four weeks of intervention (72). In Parkinson´s disease, probiotics could be used to improve bowel movements, a significant albeit secondary symptom of the disease (73).

Furthermore, probiotics improved both quality of life and inflammation markers in patients with multiple sclerosis, though the impact on clinical symptoms was limited (74).

Probiotics in oncology

While probiotics are not at the forefront of cancer treatment regimens, their immunomodulatory characteristics and usage for specific clinical symptoms led to further investigations regarding their potential as supportive additives in standard cancer treatments.

In colorectal cancer patients, pre- or perioperative probiotic and symbiotic application significantly reduced the number of complications during and after surgery, quality of life as well as the duration

of hospitalization (75). Probiotics also improved diarrhea associated with radiation and chemotherapy (76,77).
In patients suffering from oral mucositis as a side effect of cancer treatment, oral suspensions or rinse solution containing probiotic strains were shown to significantly improve the incidence and severity of this complication, and should be considered as an alternative or additive approach to medical or other therapies (78).
Another systematic review demonstrated that probiotics improved overall survival when used in addition to standard immune checkpoint inhibitor therapies, especially in non-small cell lung cancer patients. However, the meta-analysis was conducted based on six RCTs in total, necessitating further research on this vital topic (79).

In summary, research on probiotics is still growing and gaining traction as a possible mono- or additive therapy for a wide variety of diseases and disorders. However, in order to investigate the impact of probiotics in these conditions, one needs to quantify the microbiome and its effects in measurable ways.

1.4.3 Quantifying the microbiome

Microbiome research surged in the 2010s, mostly due to technological breakthroughs, enabling fast and efficient DNA sequencing from a multitude of sources within a single polymerase chain reaction (PCR) test cycle. Prior to this approach, sequencing thousands, let alone tens of thousands of DNA sequences from a single individual was prohibitively expensive and time consuming. The technological advancements did not end there and nowadays, there are numerous different ways to measure the quality and quantity of the gut microbiome. But first, a short introduction into microbiome terminology is necessary.
When discussing microbiome samples, diversity metrics are used for comparison. Importantly, there are two main types of diversity used in this approach: alpha and beta diversity.

Alpha diversity
Alpha diversity describes the variability of different species within the same environment/host. For example, environment A has five different species of bacteria, while environment B has only two. In this example, environment A is more diverse than environment B.

There are different metrics and methods of comparing alpha diversity. The simplest is comparing the richness, aka the amount of species present in each environment, as shown above. The Chao1 index is a common metric of richness and counts all unique species within a sample and corrects for the number of observed species, taking into account that not all species present can be observed (80). This approach has the limitation of not taking into account the distribution of the specific species within a sample.

Therefore, evenness is another measure of alpha diversity in microbiome research. To use another example, environment C and D both contain 15 bacteria from five different species in total. Environment C has three bacteria from each species, while environment D has 11 bacteria from the first species and one bacterium from all other species present. In this example, both environments have the same richness, but environment C is more evenly distributed between the species, hence it is considered more diverse (80).

Alpha diversity is therefore considered a combination of both richness and evenness. There are two main indices used for alpha diversity metrics. The Shannon index counts all unique species within a sample and takes into account their distribution within the sample. The Simpson index on the other hand represents the probability that two bacteria present are not the same. While both take into account richness and evenness, Shannon index is more suitable when there are many bacteria with a low abundance within a sample, while the Simpson index is more dependent on the evenness of a sample (80).

In order to compare richness and evenness between samples and/or individuals however, it is vital to account for the total number of different DNA “reads” present. To use an example, sample E has 10.000 reads from 100 different bacteria with equal distribution, while sample F has 100.000 reads from 200 different bacteria, with many of the bacteria only present in small quantities. In this example, the richness of sample F would be greater than in sample E, however sample E would be more evenly distributed, with the same results expected with regards to Shannon and Simpson indices. In typical environmental or stool samples, several hundred thousand or even more reads can be detected, and the more reads are sought out, the more different species are discovered in the sample. However, this skews analysis and makes comparisons difficult. Since environmental samples such as soil samples or stool samples can never encompass the entirety of the environmental microbiome, read counts are not indicative of the general richness, evenness or abundancy of microorganisms in the respective environment (81).

Therefore, quality control measures need to be implemented. First, low quality reads (e.g. reads of only 50 bases, not sufficient for 16S DNA identification, see below) need to be excluded from

the raw read files. Next, the samples need to be normalized in order to maximize comparability and minimize confounding factors, such as read abundance in a sample.

Several different methods exist to accomplish this goal, however the most common one is rarefaction. By extracting the same number of reads from each sample randomly, Shannon and Simpson indices can be directly compared between the samples, as all samples now have the same read depth. The sample depth can be adjusted at will up to the maximum number of reads per sample, resulting in rarefaction curves, which show the number of detected species dependent on the total number of reads screened. A confidence interval is included, and two samples (or groups) can be compared using their rarefaction curves as a sign of available diversity within the samples. The limiting factor of the rarefaction is the lowest maximum read count of the available samples. For instance, there are ten samples to be compared. Nine have a read depth of over 200.000, while the tenth sample only has 50.000 reads in total. Therefore, the maximum rarefied read count to compare all ten samples is 50.000 in this case (81,82).

Beta diversity

While alpha diversity looks at diversity within the same sample, beta diversity explores the variations in microbial composition by comparing two or more different samples directly. Many different approaches exist to highlight comparisons, though there are only a few methods used commonly.

There is for example the Jaccard distance between two samples, highlighting if the same species is present in both samples, without taking into account the abundance of the species in each sample. It can range from 0 to 1, with 0 meaning that there is no overlap between microbiota and 1 meaning that the microbial composition is identical, while not taking into account abundance differences (83).

Another metric ranging from 0 to 1 is the Bray-Curtis distance. In contrast to Jaccard, it takes abundance into account, with 0 meaning that both samples share the same species with the exact same abundances, while 1 means that there is no overlap at all (83).

The main way of highlighting beta diversity though is through principal coordinate analysis (PCoA). By this process, samples are assigned coordinates in a multidimensional space based on their features, the bigger the distance, the more different the samples are. This makes it possible to visualize the differences in PCoA plots, usually in two or three dimensions. It breaks the multidimensional space down to the two (or three) most important features when distinguishing the individual samples. These principal coordinate features are shown as axis and samples are

highlighted along these axes. The closer two samples are together, the more similar they are with regards to these two or three principal features. However, this is only a simplification of the true distance between them, as the human imagination is limited when it comes to picturing multidimensional space with sometimes dozens or more of dimensions (83).

However, the PCoA calculation is only the first step. Next, statistical analysis needs to be conducted to assess whether any differences detected are of a random nature or not. As with previous steps, a multitude of statistical options is available (84), however PERMANOVA and ANOSIM are both commonly used. While ANOSIM compares if the features of each sample are more similar between two groups than within the same group. PERMANOVA or permutational multivariate analysis of variance on the other hand works by measuring the sum of squares of distances within a group and compares it to those with other groups. Within-group and between-group variances are compared using the F test to test the null hypothesis that between-group differences are smaller than within-group differences (84).

Another method used is redundancy analysis (RDA) based on linear regression, which explores how much the variance of one set of samples is based on the variance of another set of samples. Similarly to PCoA, RDA may be used to highlight key features distinguishing two groups of samples from each other, while also taking into account their phylogenetic or ecological differences. RDA plots are often shown as two-dimensional coordinate plots, similarly to PCoA plots (85).

Further visualization of significant differences between two or more groups can be achieved with linear discriminant analysis effect size (LEfSe). This method highlights the group features/taxonomic units/genes which are most likely responsible for the inter-group variability (86).

Phylogenetic diversity

An aspect not yet mentioned regarding diversity is phylogenetic diversity (PD), which assesses the evolutionary closeness of microbiota on the phylogenetic tree, where all life is categorized based on their divergence from each other over time. PD is defined as the sum of distances between all species under investigation on that tree. To use an example, sample A and B both have ten distinct bacteria present. In sample A, these bacteria are comprised of ten subspecies of the same bacterial species, while in sample B, each bacterium belongs to a different species or even order. In this example, Shannon and Simpson indices would be identical in both samples, however sample B is more phylogenetically diverse than sample A, as the bacteria are not as

closely related to each other. PD has several important implications. An environment with high PD expresses many different functions and or modes of action, whereby every organism seeks out their own niche for survival. In an environment with low PD, several organisms might occupy the same niche for survival, thus competing with each other instead of complimenting each other. High PD environments have a higher chance of adapting to changing circumstances, as those circumstances might eliminate some niches, while the others persevere. In contrast, low PD environments might not survive similar changes in circumstance, as there are fewer occupied niches of survival, and if those are affected, the environment is severely affected or even completely destroyed (87).

Sample type

Stool samples are the most common way of obtaining gut microbiome data from individuals, as they are easily and repeatedly obtainable and do not require invasive methods. Once collected, the stool samples should be homogenized and flash frozen with liquid nitrogen and stored at -80° Celsius. Since this is not always practical or possible, and many studies rely on repeated stool collection from participants at home, many different storage protocols exist, including stool DNA preservatives added to the sample containers. In other cases, stool samples are stored at room temperature for several hours or days prior to processing. While the type of preservative and method of storage do change the microbiome makeup of the sample somewhat, room temperature storage has been shown to be comparable to the gold standard of flash freezing at -80°C immediately after collection in terms of sample quality and composition. In the case of prolonged storage prior to processing (e.g. collection at home and return after several days), lowering the storage temperature is considered as a DNA-preserving measure (88,89).

Stool samples have additional limitations besides storage. They do not provide the full spectrum of the gut microbiome composition, as many microbiota colonize the gut in the mucosa layer of the gut walls and are not represented in stool samples. In addition, mucosa microbiome compositions vary based on the region of the intestine examined, and different microbiota can be detected in the colon compared to the ileum for example. As a result, alternative methods besides stool consist of mucosal biopsy sample collection via endoscopic procedures. However, this approach is more complicated, cost-intensive and fraught with possible health complication due to the invasiveness of the procedure (89).

Areas of interest

The most common type of analysis consists of DNA sequencing. This is based on the segment of bacterial DNA coding for the 16S ribosomal RNA present in every bacterial cell. This 16S rRNA has several variable regions differing between the various taxonomic subgroups of bacteria as well as highly preserved regions identical in most bacteria and archaea. Based on the preserved regions of 16S DNA, PCR primers were designed to reproduce them as well as any variable regions close by. Based on available databases of 16S DNA sequences, the analyzed DNA can be identified down to the genus level of taxa (90,91). Using this approach, DNA sequences with a 97 % overlap or more are grouped together as operational taxonomic units (OTU). In contrast, amplicon sequence variant (ASV) is the second method of DNA classification, whereby only DNA strands with identical nucleotide sequences are grouped together, allowing for a finer distinction between bacterial taxonomy levels, even down to species or subspecies levels (92).

Microbiome DNA can also be used for metagenomics analyses, whereby not only 16S DNA is analyzed but the entire present genome. This allows for more precise differentiation of microbial taxa and also provides information regarding the metabolic processes encoded in the DNA, however this method is more expensive and results may be skewed or contaminated based on DNA fragments from the human host (91).

In addition to DNA-based approaches of microbiome assessment, messenger RNA can also be directly quantified. This metatranscriptomic approach has the advantage that it shows metabolically active microbiota (instead of potential DNA fragment contaminants) and can help with identification and the elucidation of their respective metabolic function. However, this method is very time- and cost-consuming, the samples are difficult to collect and host RNA contamination needs to be considered (91).

Both the metagenomics and metatranscriptomic approaches may be used to assess the metavirome, or viral components of the microbiome (for DNA and RNA viruses respectively). However viral genetic information tends to have a low biomass within a microbial sample, and host DNA often contains viral components, therefore careful sample preparation and quality control needs to be in place for virome assessment, as well as the financial resources to fund this expensive technique (91).

Another important pillar of microbiome research consists of cultivating live bacteria and cataloguing their genetic and metabolic information in publicly available databases. This approach allows for single-microbial isolates and the expansion of available databases, however the choice of cultivation media and the limited number of cultivatable bacteria compared to the entire sum of bacteria present remain as issues to be addressed (91).

Ideally, a combination of genomic and metagenomics should be used whenever possible to discern which microbiota are present and their impact on metabolic pathways (91).

DNA sequencing

While these additional approaches are useful and necessary depending on the research question, DNA sequencing for either metagenomics or 16S DNA amplicon sequencing is the most common approach used today for most topics under investigation. For the purpose of sequencing, the DNA segments are marked with specific barcodes, then amplified using fluorescently marked nucleotides, allowing for the rapid and ongoing identification of the whole sequence (93,94). The resulting sequences contain forward and reverse multiplied DNA as well as the barcode sequences identifying the individual DNA sequences and the primers used for amplification. These raw data are stored in test files called FASTQ files, containing both the original sequences and their quality information, such as the Phred score, highlighting the reliability of the sequenced bases (81).

Data Preprocessing

Prior to the creation of an abundance table highlighting the different OTUs (called an OTU-table), several preprocessing steps need to be taken. Samples with very low read counts need to be filtered out of analysis, lest they reduce overall reliability and misrepresent the microbial composition (81).

In a second step, OTUs with very low relative abundances of less than 0,01 percent (or in some cases 0,1 percent) as well as low quality reads, potential contaminant reads (such as those in negative controls) are removed as well to ensure that few very low quantity or quality reads do not skew the results. Depending on the selected thresholds, filtering is both an important step of quality control and a potential source of biases for erroneous results (81).

In addition, batch effects are a major potential source of bias and should be accounted for during pre-processing. Sequences from different PCR sequencing runs contain variable results due to technical settings and limitations, therefore data needs to be adjusted accordingly (81).

Next, due to the high number of zeros in the FASTQ file (as there are hundreds or thousands of OTUs with very low abundances), imputation is used to adjust for these zeros and any missing data that fell below the threshold of detection, for example due to low sequencing depth (see rarefaction). First, the distribution of data needs to account for the excess zeros, such as the zero-inflated negative binomial distribution. Next, non-parametric models, such as k-Nearest-Neighbors

imputation or random forest imputation, infer and predict missing values based on the values detected. However, this technology needs to be used very cautiously in microbiome data, as many relationships between the species are hard or impossible to predict due to their irregularity (81).
The next step consists of the normalization of samples in order to maximize comparability. Besides rarefaction, other methods of normalization exist, such as total-sum scaling or logarithmic transformations to account for very low abundances. By excluding very low-count samples during the filtering process, all other samples may be rarefied to include rarer OTUs and thereby increase the sensitivity of the study, highlighting the importance of all preprocessing decisions for the end result (81).
Once the quality control steps have been concluded, an OTU (or ASV-) table needs to be constructed. In the case of OTUs, the table is created using a process known as clustering, whereby sequences with usually 97 % overlap (the threshold may be changed depending on the research question) are clustered together to form a single OTU. Denoising is a process used to filter erroneous sequences, leaving only putatively true microbial sequences in the sample. The resulting sequences are referred to as ASV. It is also possible to combine both processes, thereby reducing the overall size of the sample and also clustering similar sequences together to form a single OTU (95).
Nowadays, software packages are used to preprocess sequence data, the most common one being the QIIME 2 pipeline software (91). It combines the above mentioned steps and forms ready to use OTU tables. These can then be analyzed regarding alpha, beta and phylogenetic diversity.

1.4.4 PCOS and the microbiome

Microbiome diversity and its connection to PCOS pathogenesis

As more and more metabolic disorders were being investigated as potential targets for microbiome modulation, so did research on PCOS as a microbiome mediated hormonal and metabolic state of imbalance grow as well. Lindheim et al. were among the first to show lower alpha diversity metrics in stool samples from women with PCOS in a pilot study in 2017. Furthermore, they identified a relative lack of microbiota from the phylum Tenericutes compared to controls, and found an (at that time) unclassified genus from the phylum Bacteroidetes, both of which were associated with PCOS signs and/or symptoms when missing in the stool samples. (96)
A subsequent publication by Torres et al. further supported the finding from Lindheim et al. By comparing clinical data and the microbiome composition from women with PCOS, women

exhibiting only PCOM, and healthy controls, the authors also found lower alpha diversity metrics in PCOS, while also identifying hyperandrogenism and in particular total testosterone levels via multiple regression analysis as the main factor contributing to this phenomenon. In addition, eight sequence variants were identified as the main distinguishers between the groups by their abundancy. These were *Porphyromonas* spp., *Bacteroides coprophilus*, *Blautia* spp., *Faecalibacterium prausnitzii*, *Anaerococcus* spp., *Odoribacter* spp., *Roseburia* spp., and *Ruminococcus bromii.* (97).

Further subsequent studies helped elucidate on the pathophysiological processes resulting in the PCOS phenotype via gut dysbiosis.

Qi et al. identified a higher abundance of *Bacteroides vulgatus* in a Chinese PCOS cohort compared to controls, furthermore showing a corresponding reduction of glycodeoxycholic acid and tauroursodeoxycholic acid in PCOS individuals. By transplanting stool samples from the cohort study into mice, PCOS phenotypes started to appear in mice who received stool samples from women with PCOS. In another step, *Bacteroides vulgatus* alone was introduced to the gut microbiome of wild-type mice via oral gavage, which in turn developed insulin resistance and a PCOS phenotype. Interleukin 22 was identified as a key cytokine reduced in PCOS mice and human individuals as a result of fewer group 3 innate lymphoid cells from the lamina propria layer in the small intestine. Interestingly, by increasing the glycodeoxycholic acid and tauroursodeoxycholic acid concentrations in PCOS mice, PCOS phenotype characteristics were partly mitigated or even reversible. However, this mitigation was not present when interleukin 22 pathways were knocked out (98).

Gulan et al. demonstrated that higher androgen exposure in utero resulted in gut dysbiosis and subsequent development of PCOS-like phenotypes as well as higher concentrations of pro-inflammatory cytokines in newborn female rats, a process which was partially reversible by early antibiotic treatment, suggesting a microbiome-mediated induction of the PCOS-like phenotype (99).

Probiotic RCTs for PCOS

To further examine the clinical application of hormone – microbiome interactions in PCOS, several RCTs involving probiotic intervention have been conducted already.

Ahmadi et al. tested a multistrain probiotic against a placebo for 12 weeks in 60 women with PCOS and found statistically significant improvement in weight, fasting glucose (FG) levels, homeostasis

model assessment for insulin resistance (HOMA-IR), triglycerides and very low-density lipoprotein levels after the intervention period in the probiotic group (100).

Jamilian et al. found significantly lower levels of total testosterone (TT), modified Ferriman-Gallway (mFG) scores, high-sensitive C-reactive protein (hs-CRP), total antioxidant capacity (TAC), glutathione and malondialdehyde (MDA) in a similar study from Iran, as did Karamali et al. (101,102). In contrast, Karimi et al. conducted an RCT with a larger sample size of 99 participants with PCOS for 12 weeks, which did not find any statistically significant differences between the probiotic and control group, except for apelin 36 concentrations, diastolic blood pressure, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) levels (103,104). Another RCT conducted in Arak, Iran found improved SHBG, mFG, hsCRP, nitric oxide, MDA and free androgen index (FAI) levels, though no significant differences in TT levels in 60 PCOS patients in total (105). Two other RCTs from Iran also reported significant improvements in glucose and hormone metabolism parameters in women with PCOS after intervention with multistrain probiotics (106,107), while a third study went further and compared four interventions consisting of pomegranate juice alone, pomegranate juice combined with a synbiotic mixture, the synbiotic mixture alone and a placebo in 92 women with PCOS in total. The authors found statistically significant improvements in both glucose and androgen metabolism endpoints in both groups using the synbiotic, while improved blood pressure and reduced systemic inflammation and OS parameters were reported in the intervention group using the pomegranate juice alone (108,109). Apart from studies conducted in Iran, more recent studies were reported in Poland, India and Germany. The Polish study used lifestyle modifying regimens in combination with a multistrain synbiotic or placebo and found improved TT levels in the synbiotic group compared to the placebo group, though both groups managed to lose weight (110). Kaur et al. opted for a 6-month intervention period comparing a synbiotic with a placebo in 102 women with PCOS and found significant improvements in almost all metabolic and hormonal endpoints (111).

Szydlowska et al. opted for a 12-week intervention with another multistrain probiotic in 50 women with PCOS and reported significant reductions in ASD, LH and thyroid-stimulating hormone (TSH) levels as well as body mass index (BMI), though no significant TT reduction (112).

The role of phytoestrogens in PCOS

Apart from probiotic intervention, phytoestrogen consumption has increasingly been found of interest in PCOS, causing interactions with the gut microbiome and thereby mitigating PCOS symptoms via their metabolites.

Phytoestrogens are estrogen-like molecules found in plant-based diets. Soybeans especially contain large amounts of phytoestrogenic substances in the form of isoflavones. Though bioavailability varies greatly depending on the genetics, microbiome composition, subject health, food source and preparation, upon ingestion of soybeans, the glucoside molecules of genistin, daidzin and glycitin get hydrolyzed into their functional aglycone counterparts genistein, daidzein and glycitein respectively. The aglycones can either be absorbed and enter the bloodstream via the portal vein pathway, or may be returned to the gut via the enterohepatic bile pathway (113).
Further metabolization via intestinal microbiota can lead to equol, a more potent and bioavailable derivative of daidzein, with a higher affinity for estrogen receptors than the original isoflavones. In addition, it can bind 5α-dihydrotestosterone and thereby exert and anti-androgenic effect as well (113).
Only 25-50 percent of human subjects have the capacity to produce equol upon soy product consumption, depending on their gut microbiota compositions, while equol-non-producers metabolize daidzein into other substances, which do not have an estrogenic effect. It may take more than one specific species of bacteria to produce equol, though the process is still under investigation (113).
Isoflavone supplementation has been investigated for its potential in mitigating PCOS symptoms. A recent review found a positive effect on ovarian function, hormone status and insulin resistance in women with PCOS after ingestion of genistein (114), while another suggested a daily dose of 50 mg isoflavone supplementation to decrease androgen levels, low-grade systemic inflammation, OS, insulin resistance and menstrual cycle dysfunction (115).
Haudum et al. could also demonstrate a negative correlation between equol concentrations and androgens levels, in addition to an improvement of the gut microbiome composition in women with PCOS after a short-term isoflavone intervention, corresponding to improved glucose metabolism metrics (116).
Despite these publications, equol remains an under-investigated molecule in PCOS, both regarding the prevalence of equol-producers in women with PCOS, as well as the potential of inducing equol-production to mitigate PCOS symptoms.

1.5 Open questions

Based on previous knowledge regarding PCOS treatment and in order to fill a gap in current therapeutic options for women with PCOS, a randomized controlled trial (RCT) with probiotic

intervention compared with placebo needed to be designed and conducted to assess the potential of probiotics to improve hormonal and metabolic imbalances. In order to do so, further work was needed to determine its methods, the endpoints needed, inclusion/exclusion criteria, recruitment speed, safety of the intervention and adherence to the study protocol.

Hence, several questions needed to be answered prior to the conduct of an RCT:

1. Which bacterial strains should be used in the RCT?
2. Which inclusion and exclusion criteria should be applied and why?
3. How many study visits are needed?
4. What is the optimal intervention period to expect a positive effect from the probiotics?
5. What is the safety profile of the used probiotics?
6. What endpoints are suitable for a probiotic intervention trial?
7. Which endpoint should be considered the primary endpoint?
8. Do PCOS phenotypes matter regarding the expected efficacy of the intervention?
9. Should women with only one PCOS criterion be included in the trial?
10. How many women need to be recruited? What is the expected drop-out rate?

Based on the answers to these questions, the RCT protocol was designed accordingly.

2 Methods and materials

2.1 Probiotic pilot trial

The first six questions were assessed by conducting a probiotic intervention pilot trial, called "ProPCO-Pilot". Its aim was to show that a probiotic intervention trial is feasible, affordable and practicable while testing the methodology for application in the larger RCT.

2.1.1 Funding

The probiotic intervention pilot trial was conducted in collaboration with the probiotic producer Institut Allergosan GmbH, Graz, Austria in preparation for the COMET K1 Center CBmed project 3.22 "Diagnostic and predictive biomarkers in disorders of fertility and metabolism". Another collaboration partner, Winclove Probiotics B.V., Netherlands, manufactured the commercial probiotic products for Allergosan used in the pilot trial. The COMET K1 Center CBmed was funded by the Federal Ministry of Transport, Innovation and Technology, the Federal Ministry of Science, Research and Economy, Styrian Department 12, Business and Innovation, the Styrian Business Promotion Agency, Salzburg and Vienna Business Agency. The Comet program is executed by the Austrian Research Promotion Agency ("Österreichische Forschungsförderungsgesellschaft", FFG)
Neither partner were involved in data collection or analysis. They were regularly appraised on the state of the trial and recruitment progress by the principal investigator and sponsor of the trial Prof. Barbara Obermayer-Pietsch, MD.

2.1.2 Study design

The study is a monocentric, open-label randomized trial, recruiting 10 women with PCOS in three treatment groups respectively, testing three different probiotics from the company Institut Allergosan, Graz, Austria. 10 participants per group allowed for some extrapolation regarding the recruitment speed, the safety profile of the probiotics used and the estimated drop-out rate, while keeping to the financial, material and time limits of a pilot trial.
Participants were allocated randomly to one of three probiotic interventions. A randomization list was created before the start of the trial, allocating all participants to one of the treatment arms.

The study nurse for the trial had access to the randomization list and informed the recruiting investigator which treatment arm the participant would be allocated to at the time of randomization (visit 2). Three study visits were conducted, with a maximum of four months between the first and last visit.
The study was approved by the ethics review board of the Medical University of Graz (vote number 30-205 ex 17/18).

2.1.3 Outcome measures

As a probiotic intervention pilot study, the most common PCOS and microbiome composition endpoints were used. In addition, equol production capabilities within each individual´s gut microbiome as well as gut barrier function were also evaluated, the former in order to examine the prevalence of equol producers within PCOS, the latter was included due to the impact of microinflammatory processes within the gut on PCOS metabolism and microbiome composition.
Therefore, these were the resulting study endpoints:

- Glucose metabolism (oral glucose tolerance test - oGTT)
    - Homeostasis Model Assessment for Insulin Resistance (HOMA-IR) index
    - Glucose area under the curve (Glucose AUC)
    - Insulin area under the curve (Insulin AUC)
- Hormonal parameters of PCOS
    - Anti-Müllerian hormone (AMH)
    - Androstenedione (ASD);
    - Follicle-stimulating hormone (FSH)
    - Luteinizing hormone (LH)
    - LH/FSH ratio
    - Dehydroepiandrosterone-sulphate (DHEA-S)
    - 17-OH-Progesterone
    - 17-β-OH-Estradiol;
    - Total testosterone (TT)
    - Free testosterone (fTesto)
- Hirsutism via modified Ferriman-Gallwey (mFG)-score
- Body weight (BMI)
- Microbiome composition and metagenomic profile (16S-RNA gene sequencing)

- Quality of life
    - PCOS questionnaire
    - Depression questionnaires (Beck´s Depression Inventory, BDI)
    - Diet questionnaires)
- Gut permeability and inflammation (experimental)
    - Urine sucrose, lactulose, mannitol concentrations after ingestion of a sugar solution containing all three substances
    - surrogate parameters: serum diaminooxydase (DAO); stool zonulin; calprotectin; lipopolysaccharide (LPS); soluble Cluster of Differentiation 14 (sCD14); bacterial DNA
- Urine daidzein and equol concentrations (experimental)

In order to exclude other conditions known to cause PCOS-like symptoms such as congenital adrenal hyperplasia (CAH), hyperprolactinemia and Cushing´s syndrome, 17α-hydroxyprogesterone (17OHP), prolactin as well as serum cortisol levels were measured but not considered study endpoints. In cases where Cushing´s disease was suspected, a dexamethasone suppression test could be conducted as an additional feature to minimize the risk of including non-PCOS hyperandrogenemic participants.

Gut permeability and phytoestrogen production were included in the study protocol and considered secondary endpoints of the trial, to be analyzed separately from the rest of the study. This work was not performed by Valentin Borzan, MD, therefore, results and discussion from these analyses are not included in this dissertation.

Clinical outcomes (such as body measurements and questionnaires) were assessed by the study investigators (in a majority of cases, by the thesis applicant Valentin Borzan, MD), while biochemical outcomes were measured by lab technicians at the Endocrinology Lab platform, Division of Endocrinology and Diabetology, Medical University of Graz. The technicians were not informed of the participants´ intervention allocation.

2.1.4 Recruitment criteria

As a test study prior to conducting a large RCT, the inclusion and exclusion criteria had to be broad enough to ensure adequate recruitment speed and the availability of suitable participants, while at the same time narrow enough to include exclusion criteria which could potentially have a detrimental impact on the results of the study, either by influencing the gut microbiome

composition independently of the study intervention probiotic, or by changing the sex hormone and/or glucose metabolism of prospective PCOS participants.

30 women with PCOS based on the Rotterdam criteria between 18 and 45 years of age were recruited at the outpatient clinic of the Division of Endocrinology and Diabetology, University Hospital Graz, Graz, Austria.

Women who had taken antibiotics or oral contraceptives up to three months prior to their screening visit were excluded from recruitment, as were women who had taken metformin up to six months before their screening, due to the potential long lasting effects of these drugs on the microbiome (117). Furthermore, nursing or pregnant women as well as women with type I diabetes, other androgen metabolism altering conditions such as CAH or hyperprolactinemia, chronic gastrointestinal conditions and/or allergies to soy products were also excluded prior to randomization.

The following parameters and threshold values were used to define the presence or absence of the Rotterdam criteria:

Rotterdam Criterion	**Parameter**	**Definition threshold for PCOS diagnosis**
Clinical HA	Modified Ferriman-Gallwey Score **mFG**	> 4 points (118)
Biochemical HA: any of the following	Total testosterone **TT**	> 0,77 ng/ml
	Free testosterone **fTesto**	> 3,18 pg/ml
	Androstenedione **ASD**	> 3,2 ng/ml
	Dehydroepiandrosterone-sulphate **DHEA-S**	> 2,75 ng/ml
Oligo-/Anovulation = Oligo-/Amenorrhea	Mean menstrual cycle length in the past 12 months	> 35 days or < 21 days
PCOM	Transvaginal ultrasound (by external gynecologists)	PCOM present

Table 2: Parameters and respective thresholds used for PCOS diagnosis based on the Rotterdam criteria. HA was defined as either clinical and/or biochemical HA being present; The measurement methods including the assays used can be found at (119); *

TT measurements used in the study were measured via immunoassay, after comparing them to TT values measured by liquid chromatography tandem mass spectrometry (LC/MS) in 113 participants. In order to include all selected participant data from the cohort, immunoassay values

were used for any further comparison, while scatterplots were created to show the correlation between the two measurements (Shown in figure 1).

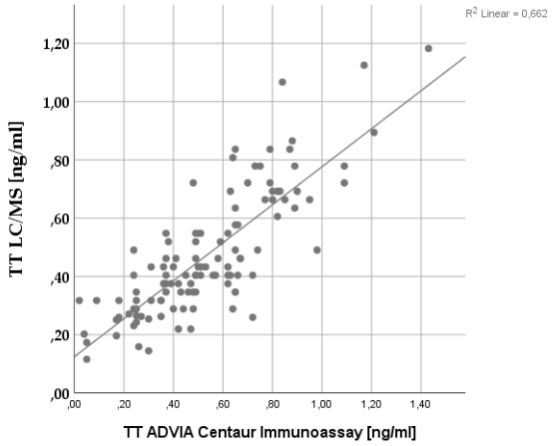


Figure 1: Total testosterone scatterplot showing the correlation between LC/MS and immunoassay measurements

2.1.5 Probiotic product:

The probiotics used were provided by Institut Allergosan, Graz, Austria, as part of our collaboration within the K1 COMET project 3.22 at CBMed Gmbh, Graz, Austria.

The probiotic products below are sold commercially in 3 g sachets containing corn starch, maltodextrin, fructo-oligosaccharide P6, inulin P2, vegetable protein and bacterial strains that differ among the respective products:

OMNi-BiOTiC® STRESS Repair: 9 bacterial strains with at least 7.5 billion organisms per 1 portion (= 3 g): *Lactobacillus casei* W56 *Lactobacillus acidophilus* W22 *Lactobacillus paracasei* W20 *Bifidobacterium lactis* W51 *Lactobacillus salivarius* W24 *Lactococcus lactis* W19 *Bifidobacterium lactis* W52 *Lactobacillus plantarum* W62 *Bifidobacterium bifidum* W23

OMNi-BiOTiC® 6: 6 bacterial strains with at least 2 billion organisms per 1 portion (= 2 g): *Bifidobacterium animalis* W53 *Lactobacillus acidophilus* W55 *Lactobacillus salivarius* W57 *Enterococcus faecium* W54 *Lactococcus lactis* W58 *Lactobacillus casei* W56

OMNi-BiOTiC® metabolic: 7 bacterial strains with at least 3 billion organisms per 1 portion (= 3 g): *Lactobacillus acidophilus* W37 *Lactobacillus casei* W56 *Enterococcus faecium* W54 *Lactobacillus acidophilus* W22 *Lactobacillus rhamnosus* W71 *Lactococcus lactis* W58 *Lactobacillus plantarum* W62

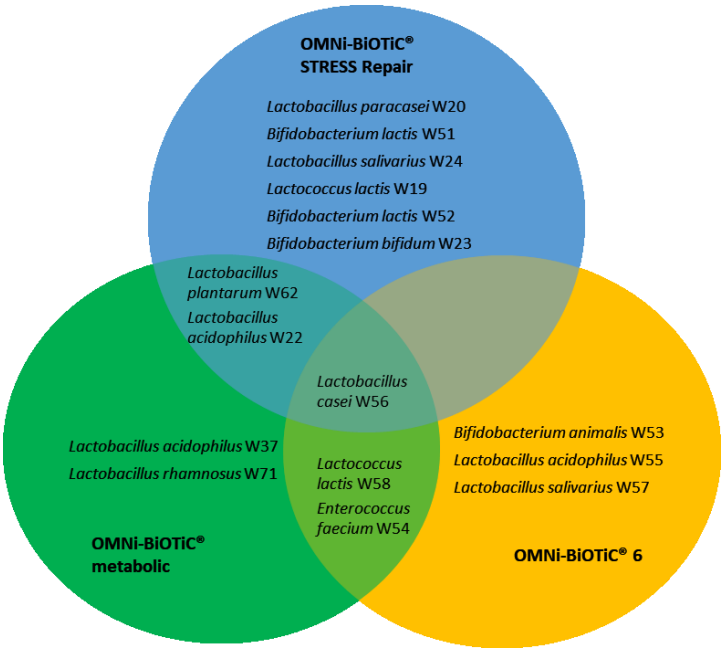


Figure 2: Venn diagram of the probiotic compositions used in our pilot trial

2.1.6 Study Visits

The eligibility of participants was evaluated at the **screening visit**. After signing the study informed consent form, a lab panel consisting of an oral glucose tolerance test and a PCOS hormone and metabolism profile was taken, the patient and family history regarding PCOS and related conditions was recorded and a physical examination was performed, documenting both clinical signs of HA as well as blood pressure, heart rate, body height and weight.

After checking for inclusion and exclusion criteria, participants were scheduled for the **first study visit**, where they were randomized and started their study intervention period. Prior to this, they were asked to perform an isoflavone challenge and a gut permeability test at home.

For the isoflavone test, participants received urine collection kits and commercially available vanilla soy milk. Two days before the second study visit, they were to collect morning urine samples, thereafter consuming the soy milk twice on that day. On the following day, they again collected morning urine samples, after which the gut permeability test was performed.

The latter was conducted on the last day before the first study visit. After ingesting a sugar solution containing sucrose, lactulose and mannitol, they collected their urine for five hours and took another urine sample. During this time, they were told not to ingest any other foods and to start drinking water after two hours in order to boost urine production. In order to evaluate the

microbiome changes before and after soy ingestion, stool samples were also collected prior and after the soy milk consumption.

The isoflavone urine samples as well as all stool samples were frozen at -18° Celsius at the participants´ homes until all samples could be returned during the first study visit, while the gut permeability test urine samples were stored at 4° to 8° Celsius and had to be returned to the lab within 24 hours after collection.

At the **second study visit**, which took place after the intervention period of approximately three months, all blood tests, physical exams and questionnaires including the at-home tests were repeated. The study design and schedule are shown in figure 3.

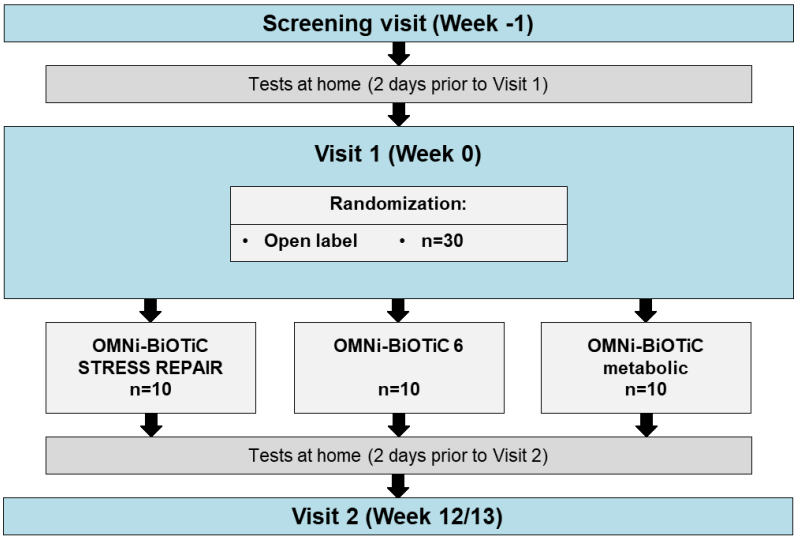


Figure 3: Study design and schedule of the ProPCO Pilot study.

2.1.7 Statistical analysis

SPSS was used for statistical analysis of the endpoint parameters, using ANOVA or Welch-ANOVA for parametric and non-parametric data respectively to detect significant differences between the three groups. Boxplots and change dot plots were created to highlight any differences in a comprehensive way.

Microbiome data was obtained from frozen stool samples collected during the tests at home, which were sent to the Institute of Clinical Molecular Biology, Kiel University, Kiel, Germany for shotgun sequencing using Illumina technology as described above. The raw reads were then analyzed via the QIIME 2 pipeline to create an OTU table in Kiel.

Together with the appropriate metadata file, the free web tool Calypso (formerly available at http://cgenome.net/calypso/, accessed in August 2020, discontinued since at least 2022) was used to assess alpha and beta diversity metrics and to create graphs, plots and figures used in this thesis.

2.2 Retrospective cohort study

While the pilot trial served to answer some of the questions relevant for the conduct of an RCT, other questions such as the determination of the primary endpoint or the evaluation of the relevance of PCOS phenotypes required a study with more than 30 participants. Therefore, a retrospective cohort study was conducted on a PCOS cohort from the in-house outpatient clinic to answer those questions.

2.2.1 Study design

Data from 800 women aged 18 to 45 years recruited between 2007 and 2015 in a PCOS cohort study at the endocrinology outpatient clinic at the University hospital of Graz were included in this retrospective analysis. The design as well as several results have been published previously (120–126), while this methodology section contains the specifics of the retrospective analysis. Data from women with only one criterion have not been published prior to Borzan et al. 2021 (119). The ethics committee of the Medical University of Graz approved of the cohort study (EC 18-066 ex 06-07).

The potential participants signed an informed consent form as part of their routine visit to the outpatient clinic, followed by blood analyses after an overnight-fast, a detailed patient history and clinical examination.

2.2.2 Outcome parameters and exclusion criteria

PCOS was diagnosed based on the Rotterdam criteria, the specific outcome parameters and thresholds for diagnosis are described in table 2 above. In addition, other causes of PCOS-typical symptoms served as exclusion criteria.

Further study endpoints consisted of sex-hormone-binding globulin (SHBG), LH, FSH, LH/FSH ratio, TSH, free thyroxine (fT_4), free triiodothyronine (fT_3), parathyroid hormone (PTH), 25-hydroxy-

vitamin D (25OHD), hemoglobin A1c (HbA1c), HOMA-IR and Matsuda index calculated from glucose and insulin levels in a 2-hour oral glucose tolerance test, total cholesterol, HDL and LDL as well as the presence of metabolic syndrome.
Similarly to the ProPCO pilot study, 17OHP, prolactin and cortisol as well as ACTH levels were determined to exclude other causes of HA.

2.2.3 Group selection and statistical analysis

Based on the Rotterdam criteria, all participants were sorted into PCOS phenotypes. In addition, a separate group consisting of women with one criterion as well as a control group without any PCOS symptoms were included. In a separate analysis, data from women with one criterion were separated as well.
If a participant could not be attributed to one of the phenotypes due to missing data, they were not included in the analysis. If the phenotype allocation was clear despite some missing data, participants were included. For example, missing information regarding PCOM was always grounds for exclusion from the analysis, while missing ASD values were not if other values of HA could be considered appropriately.
For statistical analysis and plot creation, SPSS version 25 (IBM, Armonk, NY, USA) was used. While data distribution was evaluated using Kolmogorov-Smirnov and Shapiro-Wilk tests, group comparisons with homogenous and non-homogenous variances were performed with One-way ANOVA and Welch ANOVA, as well as Bonferroni and Games-Howell post-hoc tests, respectively. Within group comparisons (for two subgroups) were tested using Student´s t and Mann-Whitney-U tests for parametric and non-parametric data respectively. Statistical significances were defined as p-values less than 0.05.
Additionally, odds ratios (OR) were determined via linear regression comparing the occurrence of insulin resistance, with PCOS phenotypes as covariates before and after adjusting for BMI and age. Furthermore, an additional OR calculation using the Rotterdam criteria as described in table 2 instead of phenotypes in order to evaluate the relevance of each criterion for IR risk.
OR were also calculated for the hyperglycemia and metabolic syndrome occurrence. For these tests, SHBG, elevated serum androgen presence and BMI were used as covariates, before and after adjusting for age and fasting insulin levels (as the latter can affect SHBG levels (127)).

2.3 Designing a probiotic intervention randomized controlled trial

Based on the results of the preparatory studies and the answers to the open questions mentioned in chapter 1.5, the pilot trial study design and protocol would serve as a scaffolding for the RCT. Depending on the open questions, minor or major changes to the study design would be necessary to adapt the RCT accordingly.

As a result, the study design of the RCT is presented as a result of the pilot trial and retrospective cohort analysis in chapter 3.5 and not elucidated further upon in this section of the thesis.

3 Results

3.1 Conducting a probiotic pilot trial

3.1.1 Recruitment for probiotic intervention trial is feasible

Over a recruitment period of one year, 60 potential participants were screened, with 30 being included in the trial. Of those, 27 completed the trial per protocol, while 3 dropped out due to withdrawn consent or due to use of antibiotics during the trial as shown in figure 4.

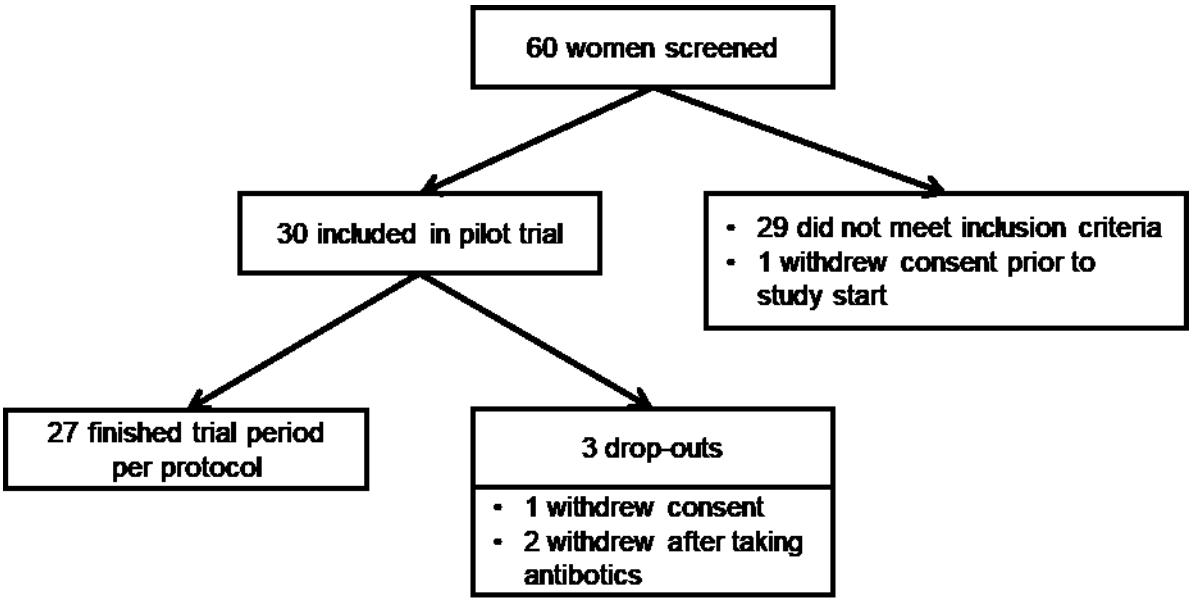


Figure 4: Study recruitment for the ProPCO Pilot study.

The 27 participants who completed the trial did not report any significant issues or difficulties regarding the trial intervention. No adverse effects attributable to the intervention products were reported, though two participants quit the trial after getting an infection which required antibiotic use in both cases. 26 participants were able to complete the self-administered tests at home (after receiving instructions from the study personnel) without any major issues, while one participant only returned the urine and stool samples associated with the isoflavone challenge test. She reported problems with collecting the urine for five hours after ingestion of the sugar solution during the gut permeability test. In addition, several participants reported mild and reversible cases of bloating and sometimes diarrhea after ingestion of the sugar solution. These cases can be

attributed to the lactulose content within. In all cases, the symptoms were self-limited and disappeared after a few hours.
No problems were reported from the blood drawings, visit schedules or the necessity to return the urine and stool samples within 24 hours after completion of the gut permeability test.

3.1.2 Comparison of outcome measures

The metabolic and hormonal characteristics of the study participants before and after intervention are displayed in table 3.

Parameter	OMNi-BiOTiC® Stress Repair		OMNi-BiOTiC® 6		OMNi-BiOTiC® metabolic		p
	Before	After	Before	After	Before	After	
Number of participants	10	9	10	8	10	10	-
TT [ng/ml]	0.45	0.48	0.37	0.35	0.45	0.47	0.963
fTesto [pg/ml]	3.07	3.78	2.08	1.99	2.58	2.35	0.347
ASD [ng/ml]	4.74	5.09	2.61	3.22	3.75	3.53	0.759
DHEA-S [µg/ml]	1.55	2.20	1.18	1.27	2.07	1.99	0.341
mFG [1]	13	16	13	8	12	13	0.628
SHBG [nmol/ml]	51.4	40.3	76.0	90.3	72.5	78.8	0.163
AMH [ng/ml]	7.98	8.82	4.04	4.89	7.99	7.23	0.869
FSH [mIU/ml]	6.86	5.45	5.53	4.57	5.06	5.57	-
LH [mIU/ml]	8.71	10.82	6.92	6.13	11.82	7.27	-
LH/FSH ratio	1.494	1.95	1.350	1.59	2.404	1.84	0.141
17OHP [ng/ml]	0.70	1.17	0.58	1.52	1.03	1.63	-
17-β-OH-Estradiol [pg/ml]	43.9	54.1	69.0	94.8	73.0	58.5	-
BMI [kg/m²]	33.1	34.1	25.1	22.5	23.3	23.2	0.083
BDI [1]	10	4	4	4	5	5	0.124
HOMA-IR [1]	2.5	3.09	2.7	1.50	1.6	1.07	0.597
Glucose AUC [mgh/dl]	237.1	248.8	214.2	184.4	220.0	187.4	0.620
Insulin AUC* [mUh/l]	134.1	120.7	55.8	52.2	123.9	66.0	0.588

Table 3: Hormonal and metabolic parameters before and after intervention; data are presented in median values; p-values were calculated with One-way ANOVA or Welch-ANOVA (depending on equality of variances) in SPSS Version 25.0; * due to hemolytic samples in all three groups, the sample size for Insulin AUC was 4, 4 and 5 for the three groups respectively

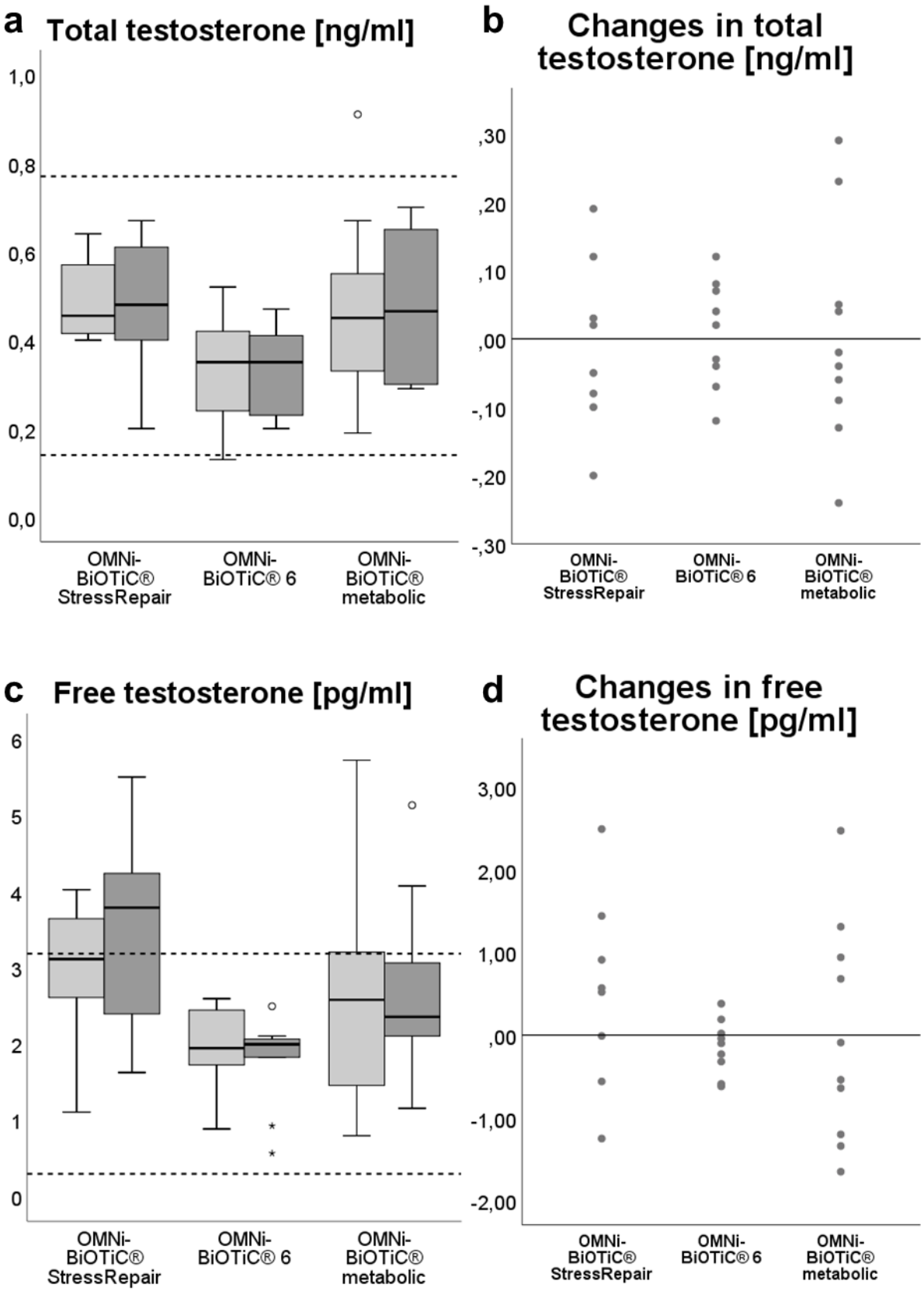
No significant differences were found between the groups after the three month intervention period. However, interesting trends can still be observed.

Of the three probiotic products used, participants taking OMNi-BiOTiC® STRESS REPAIR showed the least improvement in PCOS parameters after intervention. On the contrary, TT, fTesto, ASD, DHEA-S, mFG, AMH, LH/FSH, BMI, HOMA-IR and glucose AUC showed increased median values in this group. The one major beneficial change in the group after three months was observed in the BDI results, wherein participants reported fewer signs of depression, though both the starting and final median value were within the normal range as defined by the test.

In contrast, OMNi-BiOTiC® 6 users did show more mixed results, with declining TT, fTesto, mFG, BMI, HOMA-IR and glucose and insulin AUC median values, while presenting with increased ASD, DHEA-S and LH/FSH median levels.

Finally, the highest median decrease of fTesto was observed in the OMNi-BiOTiC® metabolic group, coinciding with lower ASD, DHEA-S, AMH, LH/FSH, HOMA-IR and glucose and insulin AUC, despite no observable median decrease in BMI.

More detailed results with regards to the specific parameters are presented in figure 5.


a
Total testosterone [ng/ml]
1,0
0,8
0,6
0,4
0,2
0,0
OMNi-BiOTiC® StressRepair
OMNi-BiOTiC® 6
OMNi-BiOTiC® metabolic
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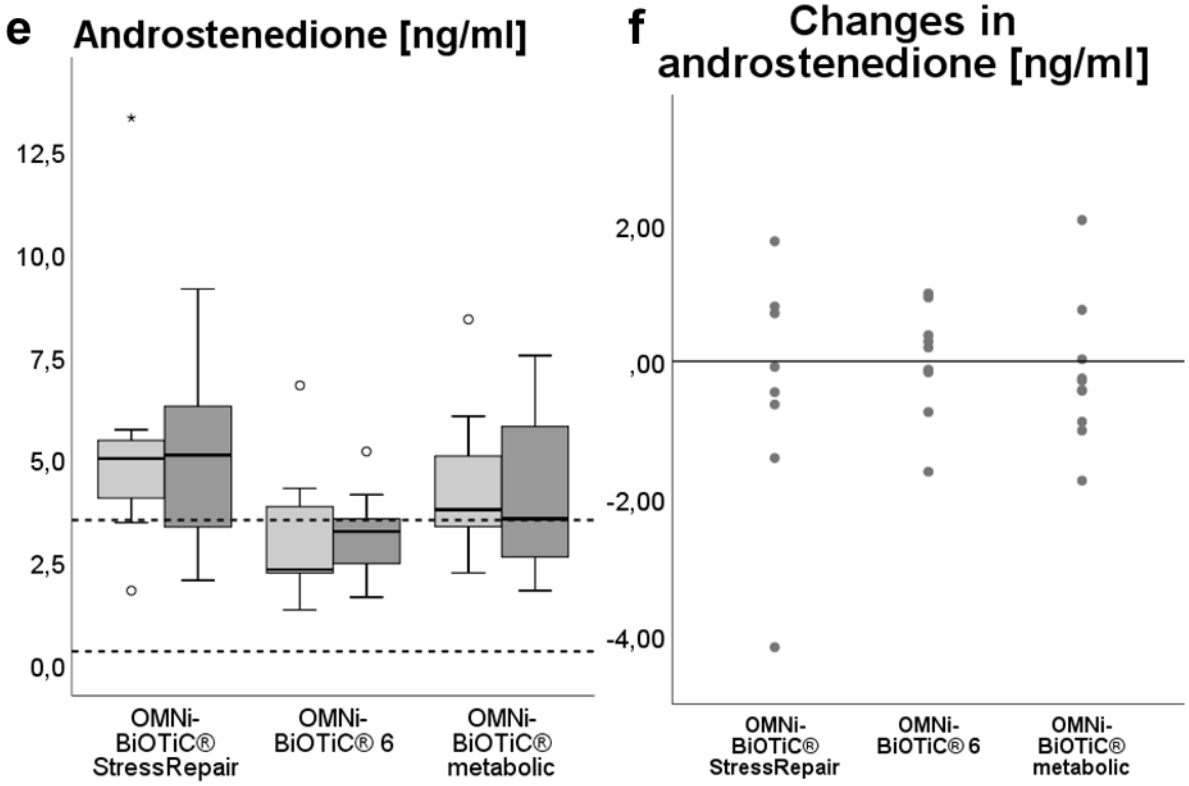
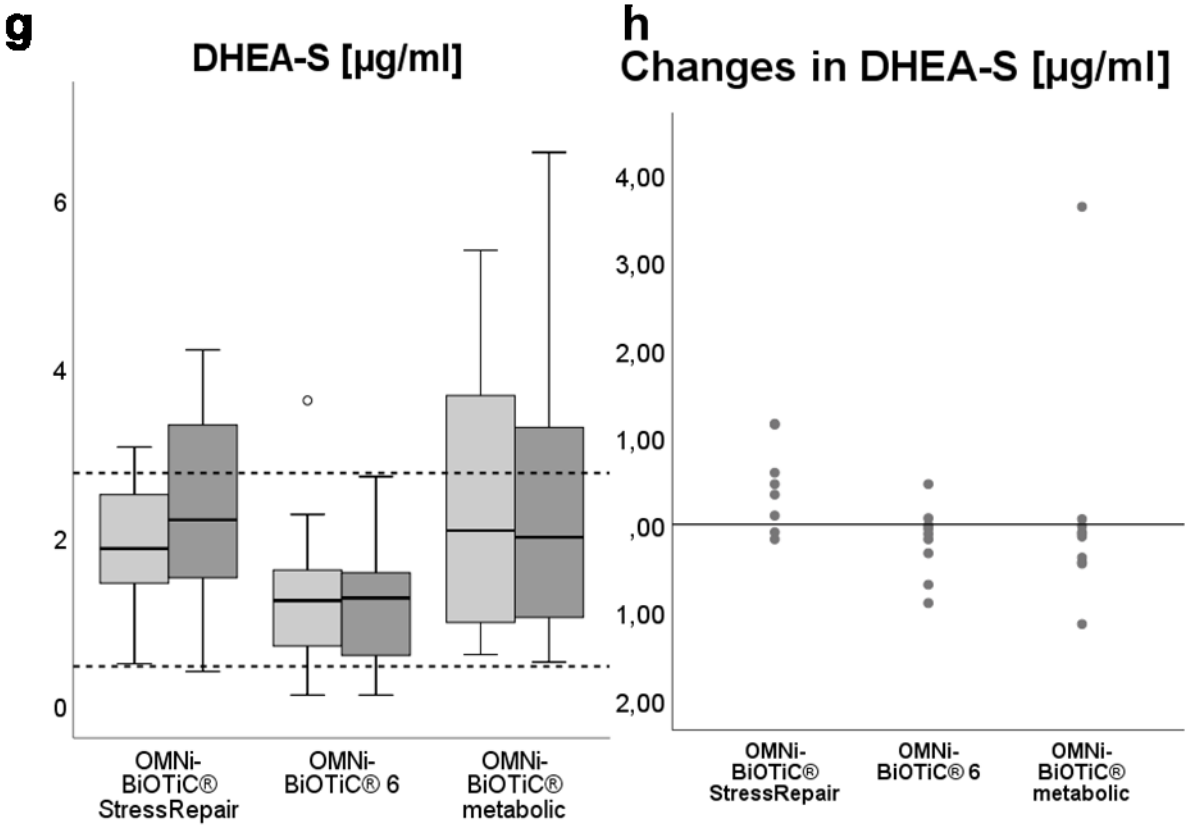
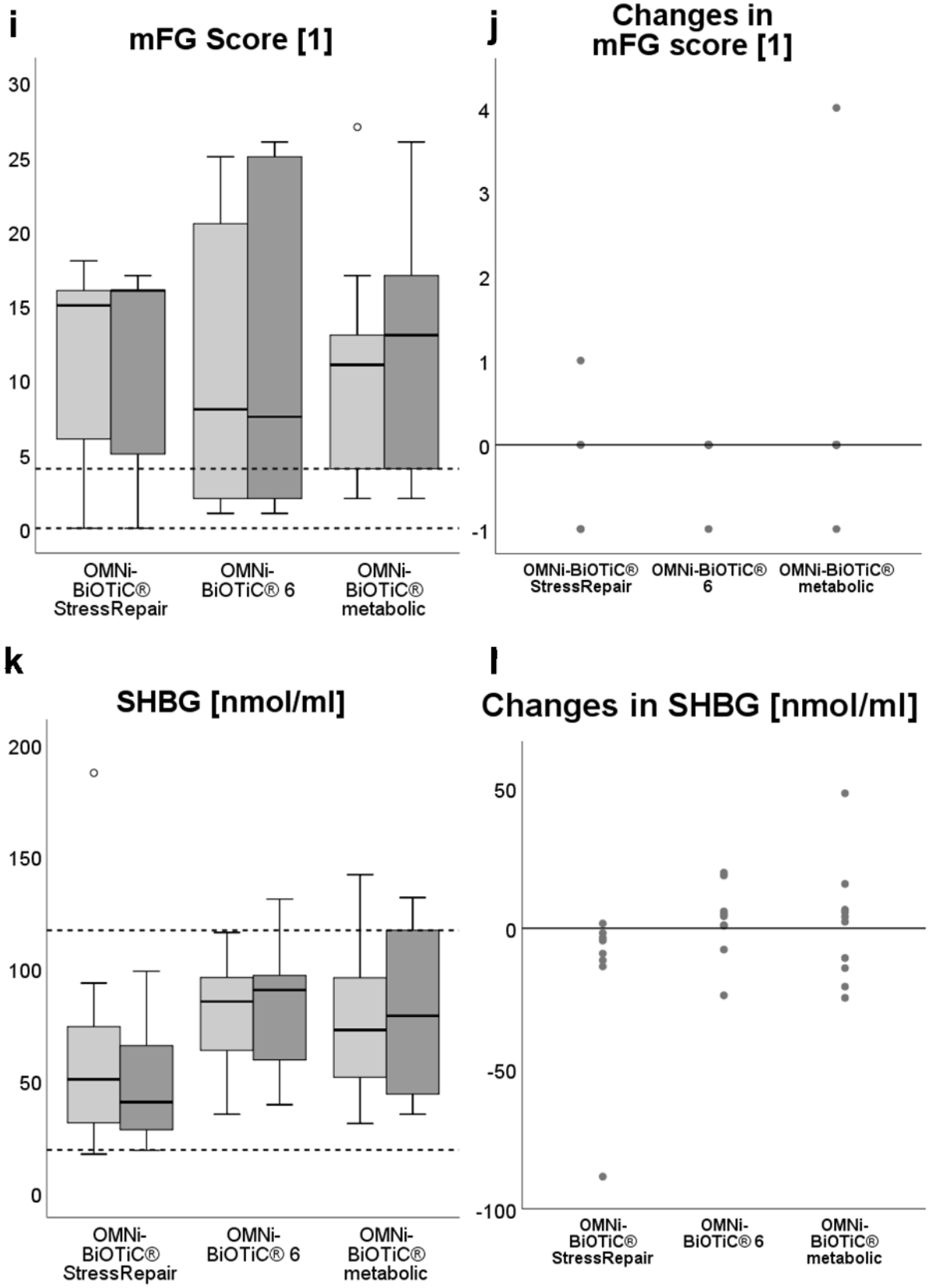
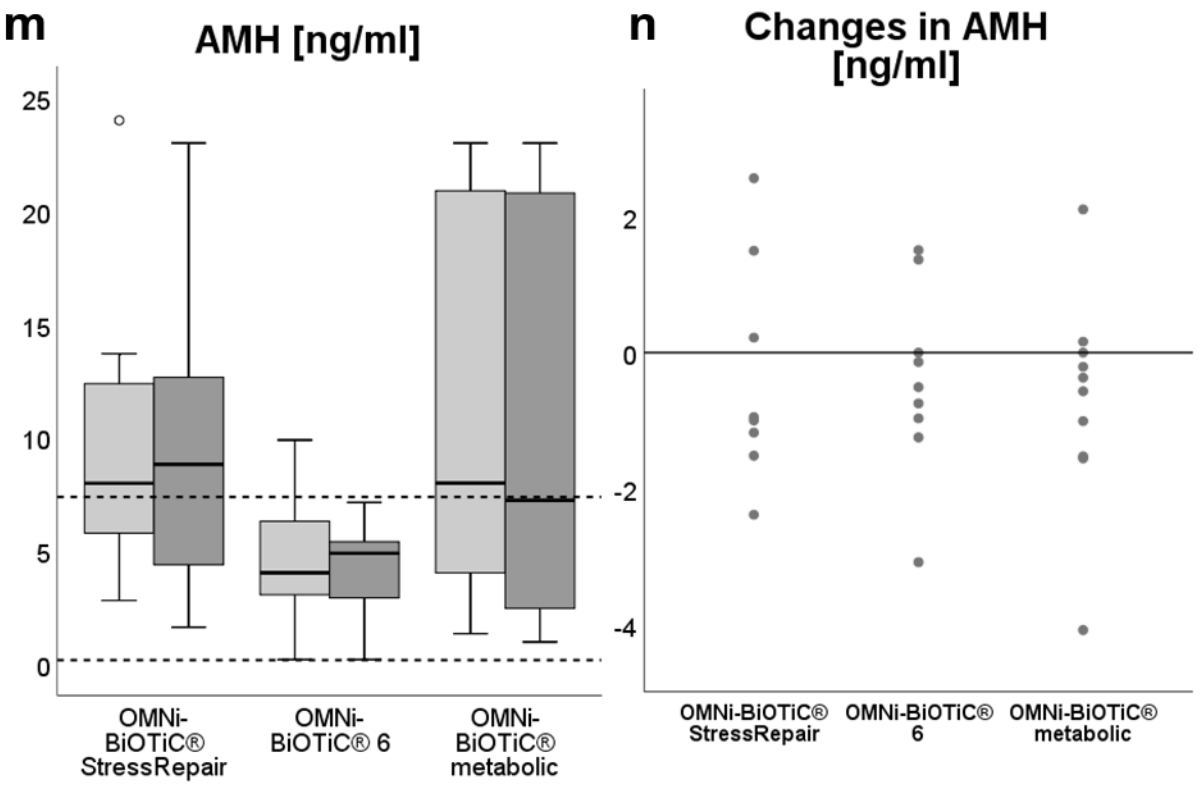
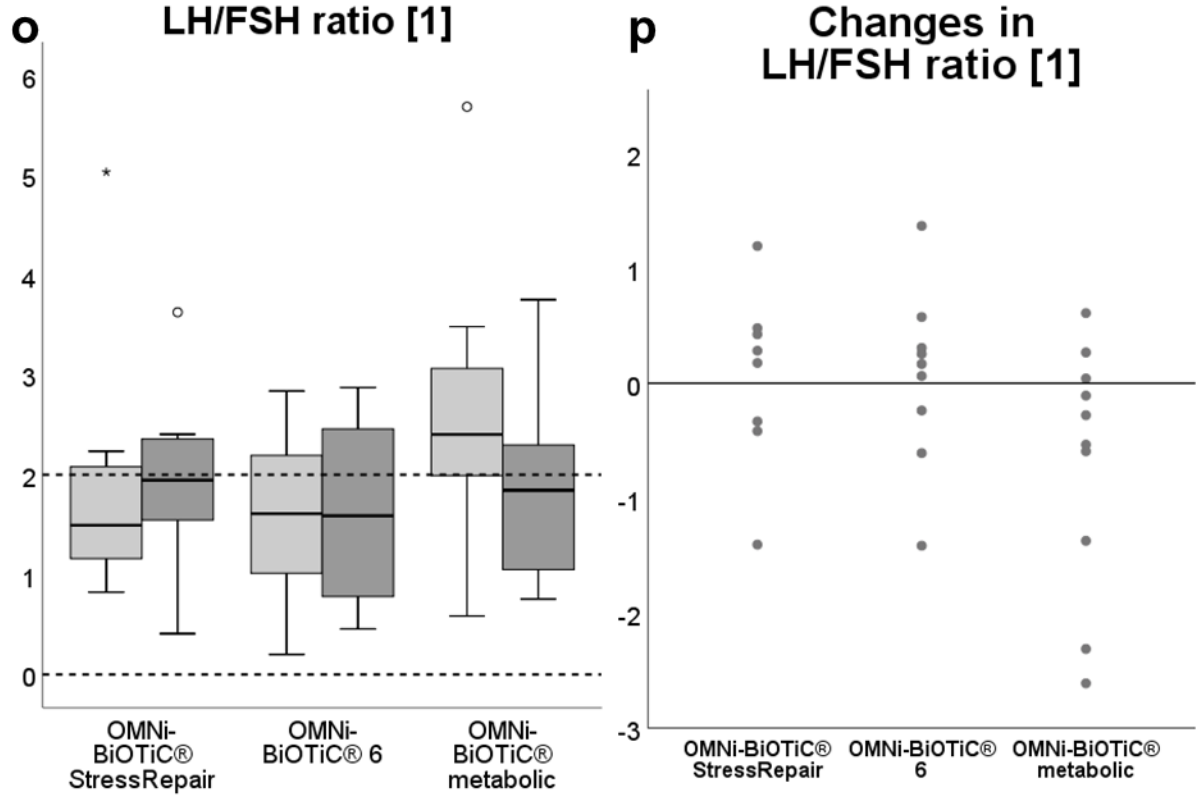
1
0
OMNi-BiOTiC® StressRepair
OMNi-BiOTiC® 6
OMNi-BiOTiC® metabolic
d
Changes in free testosterone [pg/ml]
3,00
2,00
1,00
,00
-1,00
-2,00
OMNi-BiOTiC® StressRepair
OMNi-BiOTiC® 6
OMNi-BiOTiC® metabolic

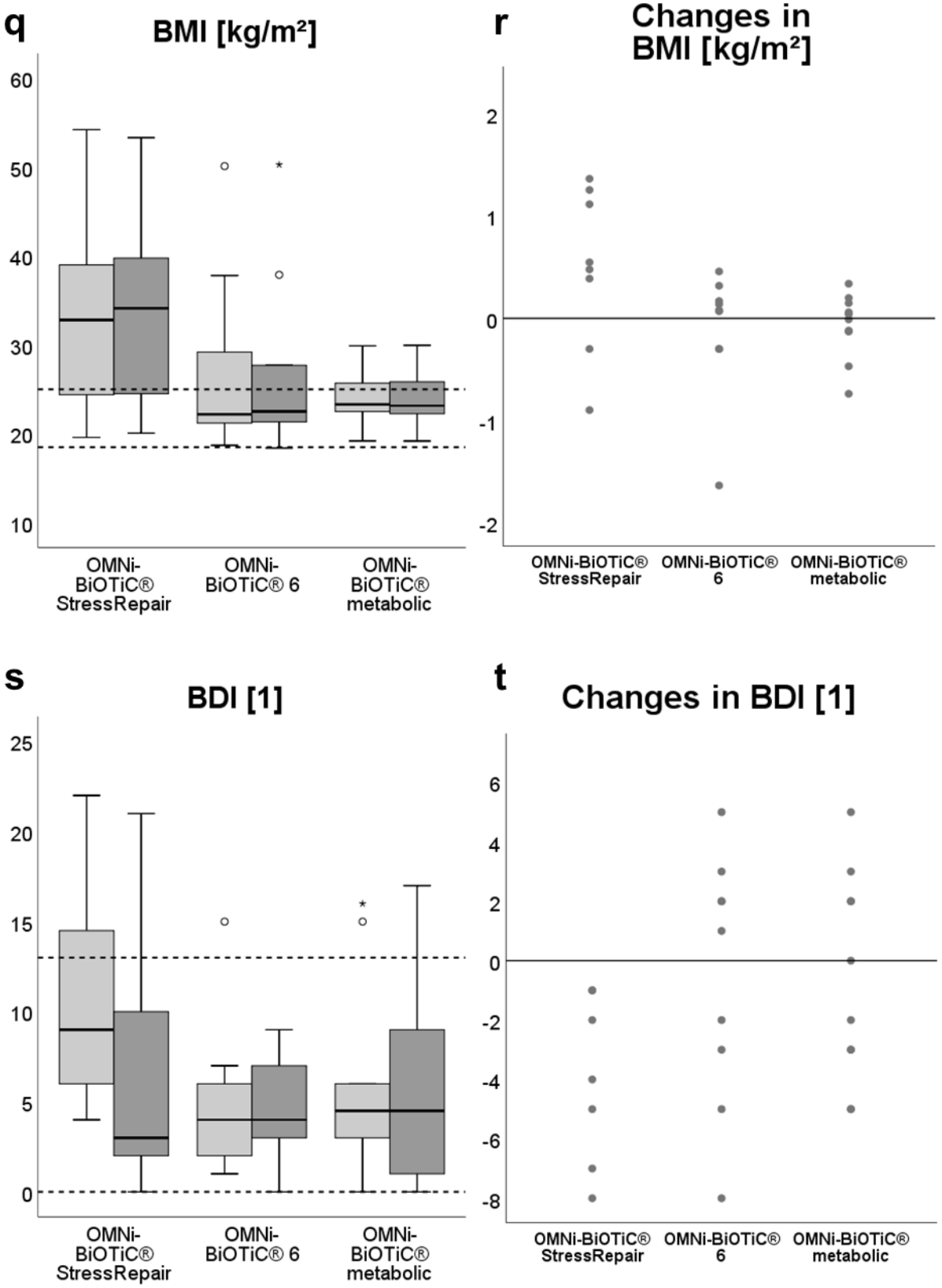

e
Androstenedione [ng/ml]
12,5
10,0
7,5
5,0
2,5
0,0
OMNi-BiOTiC® StressRepair
OMNi-BiOTiC® 6
OMNi-BiOTiC® metabolic
f
Changes in androstenedione [ng/ml]
2,00
,00
-2,00
-4,00
OMNi-BiOTiC® StressRepair
OMNi-BiOTiC® 6
OMNi-BiOTiC® metabolic


g
DHEA-S [µg/ml]
6
4
2
0
OMNi-BiOTiC® StressRepair
OMNi-BiOTiC® 6
OMNi-BiOTiC® metabolic
h
Changes in DHEA-S [µg/ml]
4,00
3,00
2,00
1,00
,00
1,00
2,00
OMNi-BiOTiC® StressRepair
OMNi-BiOTiC® 6
OMNi-BiOTiC® metabolic


i
mFG Score [1]
30
25
20
15
10
5
0
OMNi-BiOTiC® StressRepair
OMNi-BiOTiC® 6
OMNi-BiOTiC® metabolic
j
Changes in mFG score [1]
4
3
2
1
0
-1
OMNi-BiOTiC® StressRepair
OMNi-BiOTiC® 6
OMNi-BiOTiC® metabolic
k
SHBG [nmol/ml]
200
150
100
50
0
OMNi-BiOTiC® StressRepair
OMNi-BiOTiC® 6
OMNi-BiOTiC® metabolic
l
Changes in SHBG [nmol/ml]
50
0
-50
-100
OMNi-BiOTiC® StressRepair
OMNi-BiOTiC® 6
OMNi-BiOTiC® metabolic


m
AMH [ng/ml]
25
20
15
10
5
0
OMNi-BiOTiC® StressRepair
OMNi-BiOTiC® 6
OMNi-BiOTiC® metabolic
n
Changes in AMH [ng/ml]
2
0
-2
-4
OMNi-BiOTiC® StressRepair
OMNi-BiOTiC® 6
OMNi-BiOTiC® metabolic


o
LH/FSH ratio [1]
6
5
4
3
2
1
0
OMNi-BiOTiC® StressRepair
OMNi-BiOTiC® 6
OMNi-BiOTiC® metabolic
p
Changes in LH/FSH ratio [1]
2
1
0
-1
-2
-3
OMNi-BiOTiC® StressRepair
OMNi-BiOTiC® 6
OMNi-BiOTiC® metabolic


q
BMI [kg/m²]
60
50
40
30
20
10
OMNi-BiOTiC® StressRepair
OMNi-BiOTiC® 6
OMNi-BiOTiC® metabolic
r
Changes in BMI [kg/m²]
2
1
0
-1
-2
OMNi-BiOTiC® StressRepair
OMNi-BiOTiC® 6
OMNi-BiOTiC® metabolic
s
BDI [1]
25
20
15
10
5
0
OMNi-BiOTiC® StressRepair
OMNi-BiOTiC® 6
OMNi-BiOTiC® metabolic
t
Changes in BDI [1]
6
4
2
0
-2
-4
-6
-8
OMNi-BiOTiC® StressRepair
OMNi-BiOTiC® 6
OMNi-BiOTiC® metabolic

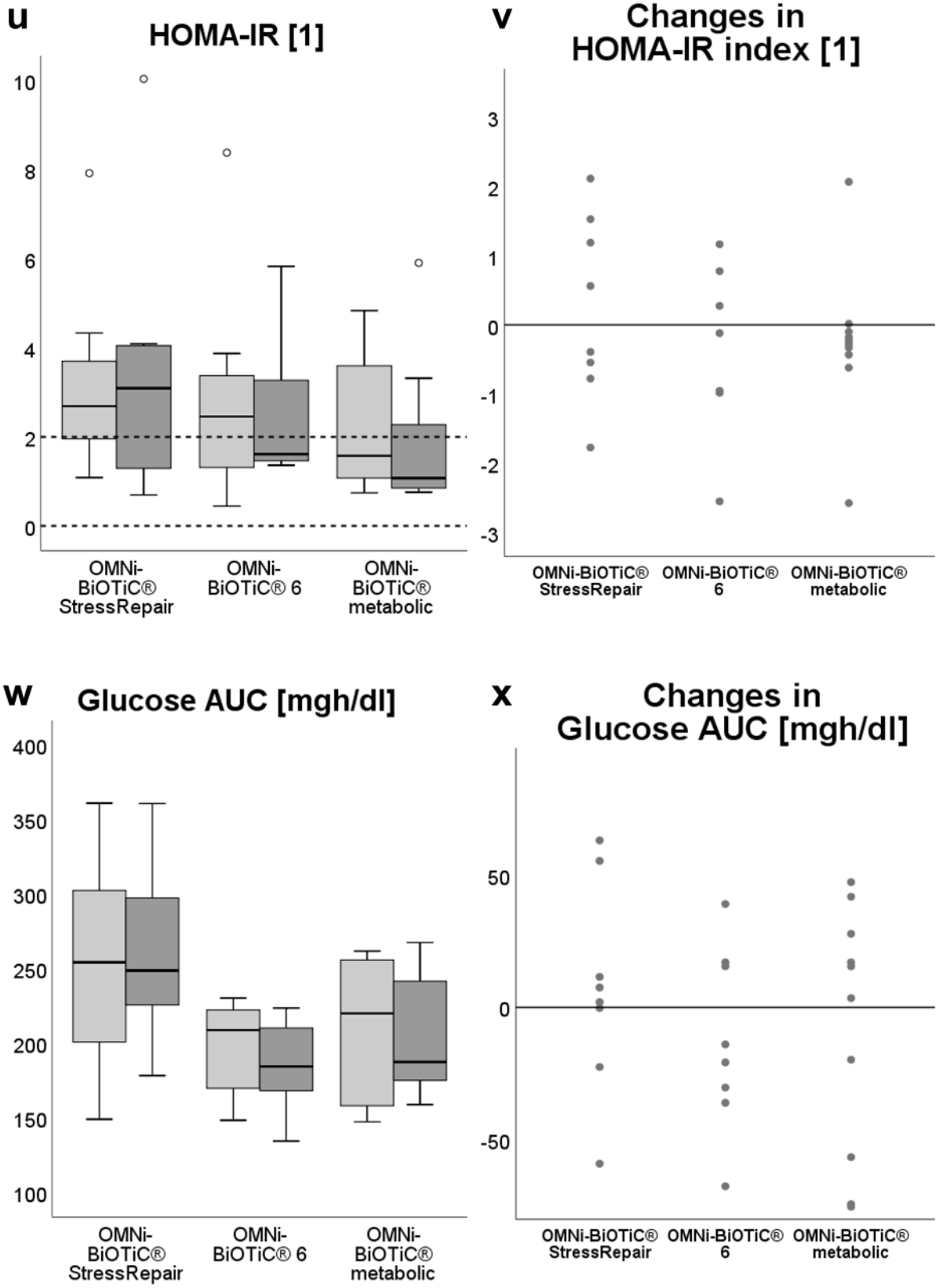

u
HOMA-IR [1]
10
8
6
4
2
0
OMNi-BiOTiC® StressRepair
OMNi-BiOTiC® 6
OMNi-BiOTiC® metabolic
v
Changes in HOMA-IR index [1]
3
2
1
0
-1
-2
-3
OMNi-BiOTiC® StressRepair
OMNi-BiOTiC® 6
OMNi-BiOTiC® metabolic
w
Glucose AUC [mgh/dl]
400
350
300
250
200
150
100
OMNi-BiOTiC® StressRepair
OMNi-BiOTiC® 6
OMNi-BiOTiC® metabolic
x
Changes in Glucose AUC [mgh/dl]
50
0
-50
OMNi-BiOTiC® StressRepair
OMNi-BiOTiC® 6
OMNi-BiOTiC® metabolic

Figure 5a-x: Boxplots and change dot plot on the left and right respectively of the main metabolic and hormonal parameters before and after intervention in each group respectively. The dotted lines in the boxplots mark the normal ranges as referenced by the Endocrinology lab platform, Medical University of Graz. The respective unit of the boxplots is described in square brackets, boxplots displaying [1] refer to parameters without any units. The figures were created in SPSS using raw data from the cohort study, therefore decimal units were separated by commas instead of points.

3.1.3 Changes in microbiome composition

In addition to hormonal and metabolic parameters, the gut microbiome compositions between the groups after the probiotic intervention were also analyzed.

Sequencing and preprocessing

Of the 27 individuals who completed the trial, all were able to return at least one stool sample for the screening and the second study visit.

However, two fastq files obtained through Illumina sequencing contained inadequate base sequences, most likely due to sequencing problems with those two samples, or due to sample deterioration prior to analysis. Therefore, two more individuals were excluded from microbiome analysis, leaving 25 viable samples which were already rarefied during preprocessing to contain at least 12015 counts and at maximum 12081 counts.

Microbiome composition results

In terms of alpha diversity, OMNi-BiOTiC® metabolic and 6 showed an increased median Shannon index, while it decreased slightly in the OMNi-BiOTiC® STRESS Repair (p=0.064). The Shannon index boxplots are presented in figure 6. There were no significant differences between the groups regarding beta diversity metrics, as shown in the PCoA plot in figure 7.

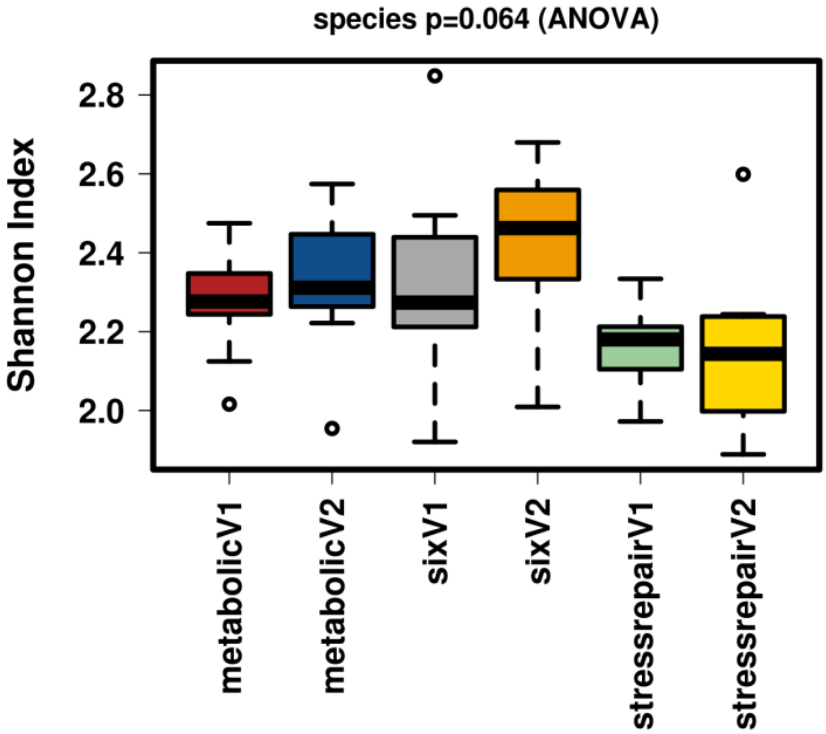


Figure 6: Shannon index boxplots highlighting before and after differences between the three intervention groups; V1: First study visit, begin of intervention period; V2: Second study visit, end of intervention period; Figure was created using the online tool Calypso (http://cgenome.net/calypso/, accessed August 20th, 2020), due to restrictions with using the tool, the three intervention groups were named as seen above instead of using the proper product names. V1 and V2 designate the study visit before and after probiotic intervention respectively

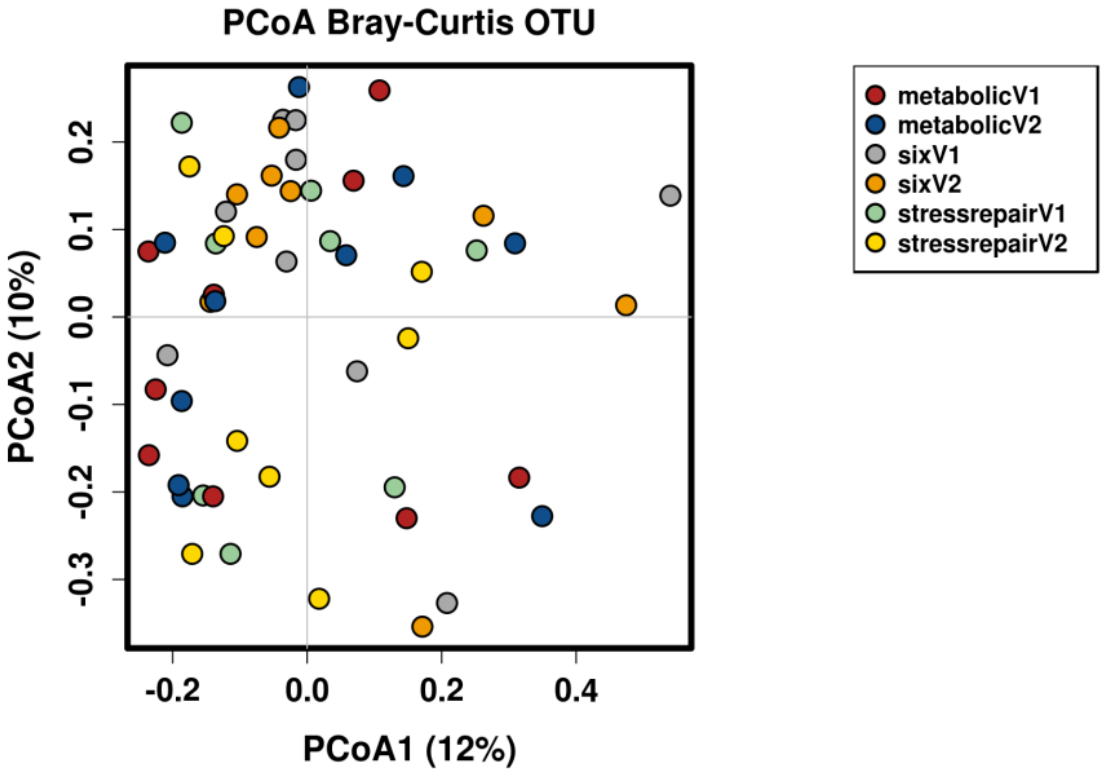


Figure 7: PCoA plot showing beta diversity differences between the groups before and after intervention; V1: First study visit, begin of intervention period; V2: Second study visit, end of intervention period; Figure was created using the online tool Calypso (http://cgenome.net/calypso/, accessed August 20th, 2020), due to restrictions with using the tool, the three intervention groups were named as seen above instead of using the proper product names

Finally, LEfSe was performed to highlight any specific OTUs distinguishing the three groups from each other. Each group had at least three OTUs which were group specific, as shown in figure 8. When comparing the effects of the intervention by performing LEfSe within each intervention group comparing the OTUs before and after intervention, no probiotic intervention strains were found using this method.

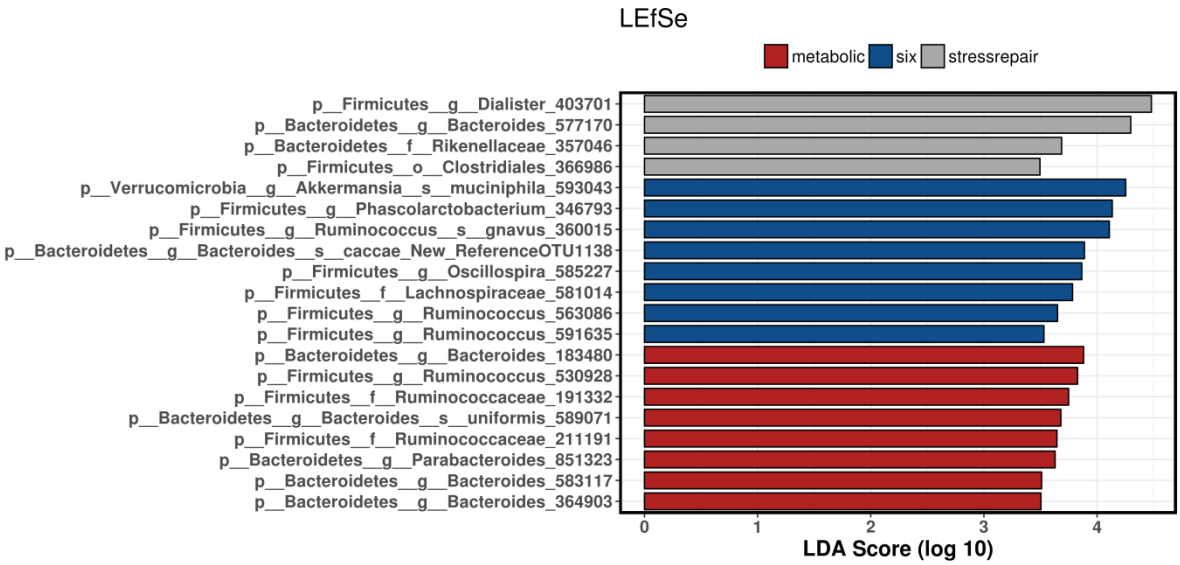


Figure 8: LEfSe analysis between the three intervention groups highlighting group specific OTUs; Figure was created using the online tool Calypso (http://cgenome.net/calypso/, accessed August 20th, 2020), due to restrictions with using the tool, the three intervention groups were named as seen above instead of using the proper product names

3.2 PCOS phenotypes matter

Based on our inclusion and exclusion criteria, data from 750 women were included in the cohort study analysis out of the 800 entries in our databank. 20 initial entries were excluded due to insufficient data, while 10 further women were using contraceptives at the time of participation, 16 women did not meet the age span inclusion criteria, 1 woman was nursing, 1 patient suffered from thyrotoxicosis, and 2 women were diagnosed with CAH instead of PCOS. Of these participants, 652 women (87 %) fulfilled the criteria for a PCOS diagnosis, 75 women (10 %) met one Rotterdam criterion (1RC), while 23 women (3 %) were assigned to the control group (Co), as they did not meet any of the Rotterdam criteria.

The participant characteristics were published in Borzan et al. and are presented here as table 4:

Outcome Parameter	A	B	C	D	1RC	Co	p
n	392	170	52	38	75	23	-
Age [y]	26.6 (22.8-30.2)	27.6 (23.2-31.1)	27.3 (23.9-29.5)	27.6 (23.1-30.4)	27.6 (22.5-33.9)	36.7 (31.6-40.9)	<0.001
BMI [kg/m²]	24.4 (21.5-29.4)	24.7 (21.6-33.2)	23.6 (21.0-31.4)	22.1 (19.9-26.3)	23.1 (20.9-28.2)	23.3 (20.8-27.2)	<0.001
TT [ng/ml]	0.62 (0.41-0.80)	0.66 (0.49-0.83)	0.49 (0.32-0.59)	0.44 (0.29-0.55)	0.46 (0.32-0.58)	0.26 (0.12-0.37)	<0.001
fTesto [pg/ml]	2.61 (1.93-3.40)	2.74 (2.05-3.93)	2.25 (1.86-3.14)	1.79 (1.43-2.16)	1.90 (1.34-2.90)	1.25 (1.02-1.67)	<0.001
ASD [ng/ml]	3.57 (2.49-4.89)	3.38 (2.52-4.68)	3.39 (2.20-4.54)	2.31 (1.79-2.81)	2.93 (2.05-4.08)	1.69 (1.37-2.14)	<0.001
DHEA-S [µg/ml]	1.97 (1.34-2.75)	2.13 (1.55-2.96)	2.09 (1.35-2.78)	1.22 (0.94-1.83)	1.90 (1.34-2.48)	1.25 (0.78-1.67)	<0.001
SHBG [nmol/l]	43.6 (29.8-64.3)	44.6 (27.1-62.5)	47.6 (28.4-68.7)	57.3 (41.4-69.7)	53.9 (38.3-77.4)	66.7 (39.5-80.4)	0.023
mFG score [1]	7 (3-11)	6 (3-11)	9 (4-12)	2 (0-3)	4 (1-10)	1.5 (0-2)	<0.001
LH [mIU/ml]	9.28 (5.29-14.00)	8.24 (4.49-13.10)	6.40 (4.64-13.21)	9.22 (3.76-12.75)	5.87 (3.24-9.31)	3.53 (2.41-8.18)	0.038
FSH [mIU/ml]	5.60 (4.27-7.06)	5.57 (4.05-7.04)	5.41 (3.80-7.60)	6.48 (5.45-8.03)	4.91 (3.35-7.15)	7.84 (4.34-9.94)	0.143
LH/FSH ratio [1]	1.64 (1.07-2.46)	1.43 (0.99-2.23)	1.51 (0.84-1.96)	1.40 (0.88-1.75)	1.12 (0.76-1.85)	0.71 (0.40-1.18)	<0.001
TSH [µIU/ml]	1.92 (1.41-2.61)	1.80 (1.23-2.39)	1.92 (1.46-2.87)	1.69 (1.02-2.48)	1.77 (1.23-2.42)	1.65 (1.16-2.09)	0.38
fT_4 [pmol/l]	14.2 (12.8-15.8)	14.5 (13.3-15.7)	14.2 (12.9-15.2)	14.1 (12.4-16.1)	14.6 (13.0-16.2)	14.7 (13.3-16.0)	0.868
fT_3 [pmol/l]	5.0 (4.6-5.4)	5.0 (4.6-5.3)	5.0 (4.7-5.4)	4.9 (4.4-5.1)	4.8 (4.4-5.2)	4.5 (4.4-5.0)	<0.001
PTH [ng/ml]	9.4 (7.5-12.9)	10.0 (7.8-14.1)	10.4 (8.2-13.3)	8.9 (5.6-11.9)	10.4 (7.9-15.7)	9.2 (7.6-15.7)	0.847
25OHD [ng/ml]	26.1 (18.5-33.1)	25.3 (16.8-31.8)	23.1 (19.0-32.0)	25.9 (17.9-30.7)	24.4 (17.9-33.3)	26.3 (18.9-34.9)	0.746
HbA1c [mmol/mol]	33 (31-35)	33 (31-35)	33 (31-36)	31 (30-33)	33 (31-34)	34 (31-37)	0.324
HOMA-IR [1]	1.6 (0.8-2.8)	1.7 (0.9-3.0)	1.7 (0.8-2.8)	1.3 (0.9-1.7)	1.2 (0.6-2.1)	0.9 (0.5-1.3)	0.215
Matsuda [1]	5.7 (3.3-10.1)	4.7 (2.8-9.1)	5.0 (3.7-9.9)	8.6 (4.8-12.1)	8.3 (4.5-15.1)	11.8 (6.3-21.1)	0.198
IR present [n (%)]	157 (40.1)	75 (44.4)	22 (42.3)	8 (21.1)	20 (27.0)	3 (13.0)	-

Hyperglycemia present [n (%)]	25 (6.4)	11 (6.5)	2 (3.8)	2 (5.3)	5 (6.7)	3 (13.0)	-
Total cholesterol [mg/dl]	175 (155-197)	175 (156-199)	167 (153-189)	173 (155-198)	174 (155-196)	179 (164-198)	0.403
HDL [mg/dl]	63 (52-75)	62 (52-77)	61 (51-74)	74 (61-82)	69 (57-83)	67 (57-82)	0.082
LDL [mg/dl]	95 (78-116)	95 (81-120)	89 (72-107)	86 (71-113)	92 (74-111)	101 (94-121)	0.119
Triglycerides [mg/dl]	72 (54-98)	78 (56-101)	70 (52-88)	64 (45-78)	59 (48-75)	58 (45-71)	0.020
MetS present [n (%)]	57 (14.5)	25 (14.7)	5 (9.6)	2 (5.3)	5 (6.7)	1 (4.3)	-

Table 4. Characteristics of PCOS phenotype A-D, patients with one Rotterdam criterion (1RC) and healthy controls. Data are presented as medians with interquartile ranges (IQR), except when specified otherwise in each row. The p-value in the last column shows the overall ANOVA/Welch-ANOVA statistical significance; BMI: Body mass index; TT: Total testosterone (measured via immunoassay); fTesto: Free testosterone; ASD: Androstenedione; DHEA-S: Dehydroepiandrosterone-sulphate; SHBG: Sex-hormone binding globulin; mFG score: Modified Ferriman–Gallwey score; LH: Luteinizing hormone; FSH: Follicle-stimulating hormone; TSH: Thyroid-stimulating hormone; fT4: free thyroxine; fT3; free triiodothyronine; 25OHD: 25-hydroxy-vitamin D: HbA1c: Glycated hemoglobin; HOMA-IR: Homeostasis model assessment for insulin resistance; IR: Insulin resistance; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; MetS: Metabolic Syndrome; Phenotype definitions: A: Hyperandrogenism (HA), Oligomenorrhea (OM) and Polycystic Ovarian Morphology (PCOM) present; B: HA and OM present; C: HA and PCOM present; D: OM and PCOM present; 1RC: Only one criterion (HA, OM or PCOM) present; Co: Control group (no Rotterdam criteria present). Cited from (119)

When allocated based on the Rotterdam criteria, the PCOS phenotypes show different trends in sex hormone and metabolic parameter levels, despite having similar criteria. Phenotypes A and B, exhibiting both HA and OM, show significant differences in androgen levels as well as BMI compared to phenotype D (without any HA signs), while phenotype C (HA and PCOM) does not. SHBG on the other hand seems to be inversely related to sex hormone levels. In contrast, no significant inter-group-differences were found when comparing thyroid hormone levels as well as 25OHD. The same is true for glucose metabolism parameters such as HOMA-IR and Matsuda indices despite promising trends, however this could be due to the non-equal group allocation and the low number of controls. A visualization of the differences between the phenotypes is shown in figure 9.

The hormonal and metabolic differences (including the trends in HOMA-IR and Matsuda indices) suggest a differing degree of symptom severity and metabolic risk between the PCOS phenotypes.

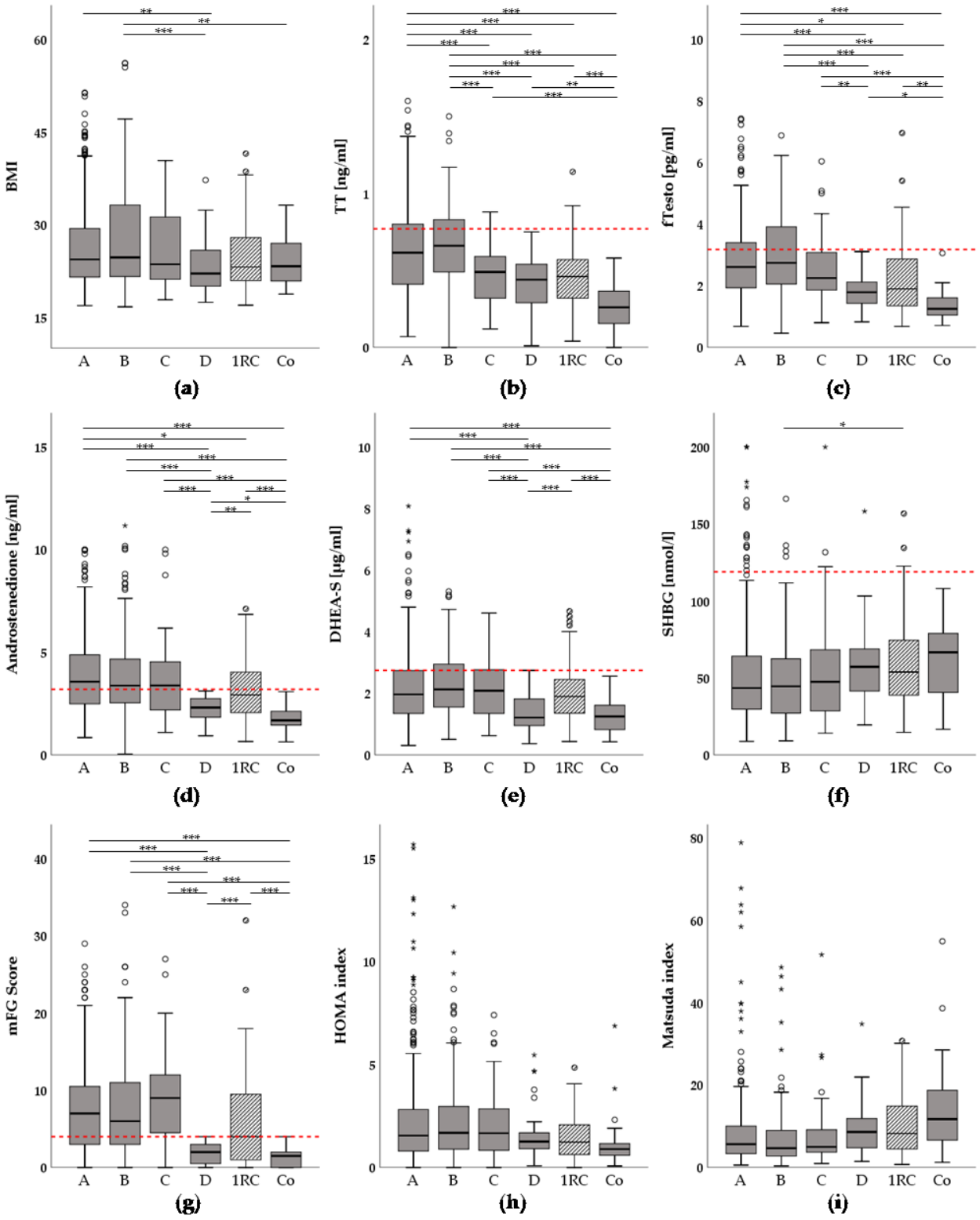


Figure 9. Boxplots showing hormonal and metabolic differences between the PCOS phenotypes A-D and the 1RC and control groups. *: p<0.05; **: p<0.01; ***: p<0.001; Red horizontal lines depict upper reference

limits for the respective parameter according to the reference laboratory; (d) further ASD outliers were: 22.05 ng/ml (group A) and 24.70 ng/ml (group B); (h) further HOMA outliers were: 29.21 (group A); 25.96 (group A); 22.67 (group B); 17.76 (group B) and 17.30 (group 1RC); (i): further Matsuda outliers were: 135.26 (group A) and 145.07 (group B); Phenotype definitions: A: Hyperandrogenism (HA), Oligomenorrhea (OM) and Polycystic Ovarian Morphology (PCOM) present; B: HA and OM present; C: HA and PCOM present; D: OM and PCOM present; 1RC: Only one criterion (HA, OM or PCOM) present; Co: Control group (no Rotterdam criteria present); BMI: Body-Mass-Index; TT: Total testosterone (measured via immunoassay); fTesto: Free testosterone; DHEA-S: Dehydroepiandrosterone-sulphate; SHBG: Sex-hormone binding globulin; mFG score: Modified Ferriman-Gallwey score; HOMA: Homeostasis Model Assessment; Cited from Borzan et al. 2021

3.3 Hyperandrogenemia is associated with insulin resistance

Within the group of women with one Rotterdam criterion, 4 individuals had PCOM, 14 women exhibited OM, while 57 women presented with HA. Of these, 13 had biochemical HA, 22 had clinical HA, and 22 women had combined HA.

In assessing the metabolic parameters between women with and without HA, no significant differences could be found. However, when we compared women with and without biochemical HA (hyperandrogenemia), individuals with elevated sex hormone levels showed significantly higher levels of not only LH, TT, fTesto, DHEA-S and, but also significantly higher HOMA indices. Inversely, women with biochemical HA showed significantly lower FSH and SHBG levels. No significant differences could be found in BMI, mFG score or the lipid status (seen in table 5).

Parameter	Hyperandrogenemia present	No Hyperandrogenemia present	p
n	35	40	-
Age [y]	27.6 (22.5-33.9)	36.7 (31.6-40.9)	0.274
BMI [kg/m²]	23.1 (20.9-28.2)	23.3 (20.8-27.2)	0.530
TT [ng/ml]	0.46 (0.32-0.58)	0.26 (0.12-0.37)	0.005
fTesto [pg/ml]	1.90 (1.34-2.90)	1.25 (1.02-1.67)	<0.001
ASD [ng/ml]	2.93 (2.05-4.08)	1.69 (1.37-2.14)	<0.001
DHEA-S [µg/ml]	1.90 (1.34-2.48)	1.25 (0.78-1.67)	<0.001
SHBG [nmol/l]	53.9 (38.3-77.4)	66.7 (39.5-80.4)	0.010
mFG Score [1]	4 (1-10)	1.5 (0-2)	0.656
LH [mIU/ml]	5.87 (3.24-9.31)	3.53 (2.41-8.18)	0.042
FSH [mIU/ml]	4.91 (3.35-7.15)	7.84 (4.34-9.94)	0.006
HOMA-IR [1]	1.2 (0.6-2.1)	0.9 (0.5-1.3)	0.021
Matsuda [1]	8.3 (4.5-15.1)	11.8 (6.3-21.1)	0.092
Total cholesterol [mg/dl]	174 (155-196)	179 (164-198)	0.231*
HDL [mg/dl]	69 (57-83)	67 (57-82)	0.910*
LDL [mg/dl]	92 (74-111)	101 (94-121)	0.072*
Triglycerides [mg/dl]	59 (48-75)	58 (45-71)	0.884*

Table 5. Group 1RC (women with only one Rotterdam criterion) group characteristics and comparisons. p-values were derived from Mann-Whitney-U tests; *: Student´s t-test instead of Mann-Whitney-U test was used for analysis due to normally distributed data; BMI: Body-Mass-Index; TT: Total testosterone (measured via immunoassay); fTesto: Free testosterone; ASD: Androstenedione; DHEA-S: Dehydroepiandrosterone-sulphate; SHBG: Sex-hormone binding globulin; mFG score: Modified Ferriman-Gallwey score; LH: Luteinizing hormone; FSH: Follicle-stimulating hormone; HOMA-IR: Homeostasis model assessment for insulin resistance; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; Cited from Borzan et al. 2021 in slightly adapted form

3.4 Elevated free testosterone is an independent risk factor for insulin resistance

Since we could show a significant association between the occurrences of hyperandrogenemia and IR, we analyzed the impact of the individual PCOS phenotypes as well as the PCOS criteria per se on the risk of developing IR via logistic regression analysis.

We found women with phenotypes A, B and C to have a significantly higher probability of IR development compared to the control group (OR 4.45, p=0.017; OR 5.32, p=0.009; OR 4.89, p=0.020 respectively). No significant results were found by comparing phenotype D (OR 1.78) and

1RC (OR 2.48) with the control group respectively. However, after adjustment for age and BMI, no significant differences between the phenotypes and the control group could be found.

Therefore, instead of comparing phenotypes, we compared the individual presence of elevated androgens (as dichotomous variables) as well as the other PCOS criteria PCOM and OM as covariates for IR risk. Counterintuitively, before age and BMI adjustment, TT showed a decreased risk of IR occurrence when elevated (OR 0.60, p=0.020), while fTesto was the only variable showing a significantly increased risk of IR development (OR 4.35, p<0.001). By adjusting for age and BMI, the lower risk of IR development with elevated TT levels was no longer significant (OR 0.66), while fTesto (OR 1.95, p=0.006) and additionally ASD (OR 1.81, p=0.006) still serve as independent risk factors for the development of IR.

In order to assess whether this is a PCOS-specific result or if women with only one criterion are also affected, we repeated the analysis for the 1RC group only and found fTesto to significantly increase IR risk (OR 21.84, p=0.013) before adjusting for age and BMI. However, after the adjustment, BMI remained the only significant risk factor, with an OR of 1.17.

Contrasting our findings regarding IR risk, hyperglycemia was not shown to be significantly impacted by elevated androgen levels or other PCOS criteria, with BMI being the only independent influencer of hyperglycemia before (OR 1.10, p<0.001) and after (OR 1.08, p=0.002) adjusting for age and fasting insulin (I0).

Interestingly, the risk of developing metabolic syndrome (MetS) was significantly impacted by elevated fTesto (OR 2.04, p=0.034), SHBG (OR 0.97, p=0.007) and BMI (1.19, p<0.001). After age and I0 adjustments, fTesto only trended towards significance (OR 1.83, p=0.093), while SHBG (OR 0.96, p<0.001), BMI (OR 1.12, p<0.001), age (1.05, p=0.046) and I0 (OR 1.05, p<0.001) had significant ORs of influencing MetS occurrence.

As a results, metabolic risk profiles can be defined based on the number and type of Rotterdam criteria present in each women with established or suspected PCOS, as shown in table 6.

	Classical PCOS phenotypes				Women with one PCOS criterion		
Symptom	**A**	**B**	**C**	**D**	Only HA	Only OM	Only PCOM
Polycystic Ovarian Morphology (PCOM)	++	-	+	+	-	-	+
Oligo-/Amenorrhea (OM)	++	++	-	+	-	+	-
Hyperandrogenism (HA)	++	++	+	-	+	-	-
Insulin resistence	++	++	+	-	+	-	-
Risk of metabolic comorbidities	+	+	+	-	(+)	-	-
PCOS Definition							
1990 NIH	✓	✓					
2003 ESHRE/ASRE (Rotterdam conference)	✓	✓	✓	✓			
2006 AE-PCOS	✓	✓	✓				
2012 NIH	✓	✓	✓	✓			

Table 6: PCOS symptoms and definitions based on clinical expression and severity; ++: severe clinical expression; +: medium to mild expression usual; (+): mild expression is possible; (-): no expression; Red hues signify high overall symptom severity, yellow hues signify intermediate symptom severity, green hue represents low symptom severity; NIH: National Institute of Health; ESHRE: European Society of Human Reproduction and Embryology; ASRE: American Society of Reproductive Medicine; AE-PCOS: Androgen Excess & PCOS Society; translated from German and cited from (17)

3.5 Successful completion of the main goals

Both the pilot and cohort studies combined were able to successfully address the main questions regarding the design of a large probiotic intervention RCT.

1. Which bacterial strains should be used in the RCT?

There are a multitude of different probiotic strains available on the market, many of them similar to each other and yet distinct. Due to restrictions in bacterial cultivation, most gut bacteria with benefits to metabolic or hormonal pathways remain off-limits in human intervention trials. Through

a cooperation with Institut Allergosan GmbH, Graz, Austria, three different commercially available multi-strain probiotic products were available for the pilot trial: OMNi-BiOTiC® STRESS Repair, OMNi-BiOTiC® 6 and OMNi-BiOTiC® metabolic. Some probiotic strains were used in all three products, such as *lactobacillus casei* W56, while some were specific to each of the three products. During the pilot trial, the participants taking OMNi-BiOTiC® STRESS Repair showed the least positive results out of all three groups after intervention. As can be seen in figure 5, most participants showed worse hormonal and metabolic values than prior to intervention. As many of the endpoints in PCOS are connected with each other via vicious cycles (see PCOS pathophysiology), it is possible that e.g. an increase in weight over time lead to increased androgen levels and higher rates of IR, and vice versa.

OMNi-BiOTiC® 6 and OMNi-BiOTiC® metabolic showed very similar results after the intervention period, leaving both as the potential probiotic of choice for the RCT. In the end, OMNi-BiOTiC® metabolic showed slightly better improvements in hormonal parameters, especially in free testosterone levels as the planned primary endpoint and determinant of the statistical power and sample size calculation of the study (see below). Therefore, OMNi-BiOTiC® metabolic was chosen as the probiotic product that would be used for the RCT.

2. Which inclusion and exclusion criteria should be applied and why?

In the pilot trial, inclusion criteria were defined based on the Rotterdam criteria, as defined in table 2. This decision was based on the recommendations as set by the 2003 ESHRE/ASRE Rotterdam conference and the 2012 NIH recommendations (1).

In contrast, use of contraceptives or metformin were defined as exclusion criteria due to their impact on PCOS pathomechanisms. In addition, metformin use has been shown to significantly alter microbiome compositions of its users, as has the use of antibiotics (128,129). Therefore, both occurrences were defined as exclusion criteria. While these exclusion criteria were detrimental to the recruitment speed of the trial, including such participants would not be scientifically and ethically sound knowing they might impact the outcome of the trial. In retrospect, there were many other medications and/or medical conditions which might impact gut microbiome composition and quality, as gut bacteria are involved with many different processes and metabolic pathways. As a result, further exclusion criteria needed to be defined for the RCT to address this loophole, such as use of proton pump inhibitors, history of major gut surgeries or history of gut cancers.

A detailed patient history, physical examination, blood and urine analysis was performed at the screening visit to properly differentiate between women who could participate and women who could not.

3. How many study visits are needed?

The three study visits in the pilot trial were shown to work well to obtain all information and conduct all necessary study procedures. The screening visit served to evaluate the study criteria, while the first study visit ensured the return of the stool and urine samples before randomization and the start of the intervention period. The second study visit marked the end of intervention and all analyses from the screening and first visits were repeated for comparison of the study endpoints. However, scheduling difficulties arose when trying to set up the dates for the second visit three months in advance. Due to the age restriction in the inclusion criteria, most participants were either students at a university or more commonly employed full- or part-time. In many cases, planning a trial visit three months in advance proved challenging, as some women did not receive their work and/or vacation schedules this far in advance. Planning was further exacerbated by the fact that most work places require their employees to be at work during the hours of the day when the trial visits could be conducted (between 7 and 11 a.m.). In most cases, the flexibility of the participants and trial investigators and study nurses made it possible to complete most visits as scheduled, with few cases where the second visit had to be rescheduled. In most cases, the new visit date was only a few days apart from the planned date, ensuring the proper trial medication intake duration. In one case, the first study visit had to be rescheduled three weeks later than initially planned, however this did not influence the probiotic intake period, as the second visit was then scheduled three months after the first.
Additional steps would be taken to improve the recruitment. First, the RCT would be promoted via social media and at gynecologists´ practices, information flyers would be left at those places for interested potential screening participants, and contact information would be given for answering open questions and scheduling screening visits. Furthermore, many participants from the pilot trial expressed interest in the follow-up RCT and asked that they be contacted once the RCT commenced with recruitment.

4. What is the optimal intervention period to expect a positive effect from the probiotics?

For the pilot trial, three months of probiotic intervention were chosen to obtain safety data, adherence to the study intervention and procedures, and to limit potential side effects of probiotics due to long-term use. For the RCT trial, experience from the pilot trial helped form expectations regarding the safety of use for the participants. Therefore the RCT would double the pilot trial intervention period to six months, with an additional six months of voluntary follow-up in order to observe potential longer term benefits of probiotic intervention.

5. What is the safety profile of the used probiotics?

All three probiotics were shown to be very reliable and easy to use during the pilot trial. Participants reported no probiotic-related adverse events during the intervention period, and most participants adhered to the recommended daily use. In order to more precisely monitor the use of probiotics, the number of leftover packages would be documented in the case report form of the RCT participants.

6. What endpoints are suitable for a probiotic intervention trial?

The study endpoints of the pilot trial reflect well the symptom severity and risk of cardiovascular and metabolic sequelae of PCOS, therefore they would be used as endpoints in the RCT as well. In addition, several experimental endpoints would be added to evaluate the impact of the probiotics on them, since there is not sufficient evidence in prior literature to expect a change in these endpoints a priori.

7. Which endpoint should be considered the primary endpoint?

The importance of the primary endpoint for a randomized controlled trial cannot be overstated. Not only is the success or failure of the trial mainly defined by showing a statistically significant positive impact of an intervention on the primary endpoint, but it is also used for the sample size calculation, therefore heavily impacting the time, resources and feasibility of trial completion. Therefore, choosing the appropriate primary endpoint parameter for the RCT might be considered the most important role of these preparatory studies.

As highlighted by the cohort study, fTesto levels play a major role in PCOS pathomechanisms. It was also identified as an independent risk factor for IR occurrence in PCOS, highlighting its relevance as a therapeutic target of intervention.
Therefore, it was defined as the primary endpoint for the RCT. In order to increase the probability of the RCT´s success, the choice of probiotic was based on the trend shown in the pilot trial, whereby OMNi-BiOTiC® metabolic was the only probiotic with a negative trend of median fTesto values in the study participants.

8. Do PCOS phenotypes matter regarding the expected efficacy of the intervention?

While PCOS phenotypes are important to consider when treating PCOS, the impact of probiotic intervention on PCOS parameters as a whole is still under researched, hence the need for the RCT. One could argue that the primary endpoint fTesto change after intervention predisposes the study to show a potential difference in the efficacy of the product based on PCOS phenotypes, however there are several points to be made for not differentiating in PCOS phenotypes during recruitment.
First, current commonly occurring PCOS phenotype separations are based on the presence or absence of specific Rotterdam criteria. And while the cohort study could show that hyperandrogenemia in particular was important to consider in PCOS metabolic risk assessment, even in phenotypes A, B and C HA is defined not by hyperandrogenemia alone, but also (or instead) by the presence of clinical signs of hyperandrogenism, such as hirsutism. Therefore, no basis exists to separate between women with high risk and those with low risk with the above mentioned phenotype definition. Both might benefit from probiotic intervention, or none may.
Second, recruiting women with PCOS fulfilling all necessary criteria for participation will be challenging enough based on the experiences of the pilot trial. The recruitment of 30 women into the trial took approximately twelve months. And while measures will be taken to increase recruitment speed, the RCT will also require a lot more participants in order to show statistical significance in its primary endpoint.
As a result, PCOS phenotypes may be assessed in sub-analyses of the trial post-recruitment, but should not be considered when recruiting women with PCOS for the RCT

9. Should women with only one PCOS criterion be included in the trial?

While the cohort study could show different risk profiles for metabolic sequelae of PCOS depending on the PCOS criteria met in women with only one criterion, the benefits these women might receive from probiotic intervention are still questionable. An argument could be made to include women with hyperandrogenemia only, as they show the highest risk profile for IR and potentially MetS occurrence, however this decision would raise several new follow-up recruitment questions and limitations.

Firstly, it is possible that women with hyperandrogenemia as their only expression of PCOS do not actually have PCOS, but another hyperandrogenemic condition, which could not be diagnosed properly as of the start of recruitment.

Second, if women with only hyperandrogenemia were included in the trial based on their risk of developing IR, then should the phenotype D (with only OM and PCOM) be excluded due to their lower metabolic risk? And how would that rule apply to the phenotype C, which only expressed HA and PCOM, but not OM and could be considered “somewhere in the middle” regarding their metabolic risk profile? The definition of this metabolic risk profile would be very arduous, not practical in the clinical setting and by definition very subjective, as these individuals did not necessarily express *any* metabolic sequelae, despite having a higher risk profile due to elevated androgen levels.

Third, the recruitment based on metabolic risk instead of strict adherence to the Rotterdam criteria would make comparisons with other PCOS RCTs very difficult, and any inclusion in meta-analyses or systematic reviews nigh impossible.

Therefore, to simplify matters and improve comparability with similar trials, the decision was made to adhere to the official PCOS diagnosis based on the Rotterdam criteria and not include any other women, independently of their metabolic risk profiles.

10. How many women need to be recruited? What recruitment speed can be expected? What is the expected drop-out rate?

During the pilot trial, 10 participants received OMNi-BiOTiC® metabolic for 3 months and showed a median (minimum-maximum) reduction in free testosterone levels of -0.3 pg/ml (-1.7-2.5). By doubling the intervention period to six months, the change in free testosterone levels was estimated at -0.6 pg/ml. The desired power to detect a difference in means of -0.6 assuming that the common standard deviation was 1.1 using a two group t-test with a 5 % two-sided significance level was 80 %, resulting in a sample size of 54 participants per treatment arm. Including a 10 %

dropout rate, 60 individuals per treatment arm were determined as necessary for the successful completion of the trial.

As for the recruitment speed, the pilot trial began with the date of the screening visit of the first patient on September 17, 2018, with the last visit of the last patient conducted on November 11, 2019. The long duration of the trial was a result of recruitment difficulties and screening failures, mostly due to the strict inclusion and exclusion criteria of the pilot trial. As antibiotic usage is very common and metformin usage in women with PCOS maybe even more so, recruiting women with PCOS who had taken neither in the last three and six months respectively proved more difficult than initially estimated.

Due to the recruitment period of the pilot trial taking approximately 12 months to complete, the RCT was scheduled for a recruitment period of two years, with the hope that the additional recruitment measures would improve the screening rate and ensure that the trial could be finished on schedule.

Feedback from the pilot study also influenced the RCT protocol design with regards to study adherence and completion of the trial, as demonstrated with the drop-out rate.

In total, three out of the recruited 30 participants did not complete the trial. One participant withdrew consent after signing the consent form due to undisclosed reasons, the second woman withdrew after the screening visit due to a contracted infection requiring the use of antibiotics, and the third woman withdrew during the intervention period for the same reason. While the first drop-out cannot be analyzed self-critically due to the unknown reason of the drop-out, the second woman could still have participated in the trial had she desired it, as she had already been recruited, though not yet randomized. The third woman also could have finished the trial, but opted to drop-out instead. Both cases lead to a motivation to improve communication with all participants, as both women could have still taken part in the trial and their results analyzed in the intention-to-treat analysis. Nonetheless, the drop-out-rate of 10 % was used to calculate the recruitment goals for the RCT sample size calculation.

Taking all of these lessons, the design of the RCT was modified to address these issues and optimize the recruitment process and improve its chance of successful completion.

3.6 Study design of a large probiotic intervention trial

Both the retrospective cohort study and the ProPCO Pilot trial served as preparation for conducting a randomized controlled trial assessing the effects of probiotic intervention in PCOS, the “ProPCO

RCT" trial. The study design and protocol were published in 2023 (130). The results and lessons learned from both studies would serve as foundations for the design and conduct of the RCT as discussed previously, with the following aims in mind.

3.6.1 Aims of the RCT

The main goal of the ProPCO RCT would be to show a statistically significant improvement of PCOS parameters in women taking the probiotic supplement intervention compared to a similar placebo. This aim served as the major determinant for the sample size calculation, the primary and most of the secondary outcome measures, as well as the overall design of the study.
However, in addition to a probiotic-vs-placebo comparison, a secondary analysis would assess the comparison of the probiotic intervention to metformin intervention within the same time frame and trial. This sub analysis would ensure that probiotic intervention would receive a direct comparison to an already established therapeutic option for PCOS, as is appropriate for conditions with already existing treatments. Should the probiotic arm prove successful, it might prove to be a feasible alternative to metformin, should the latter be rejected by patients, for whatever reason.
Additional secondary aims would be:

- Show improvement of gut barrier function through probiotic intervention in PCOS
- Show increased equol production after probiotic intervention and determine the ratio of equol producers versus non-producers in women with PCOS compared to the general population as reported by literature
- Show a colonization of probiotic strains in both the gut lumen and the mucosa layer of the intestinal wall

3.6.2 Trial endpoints

Based on the results of the retrospective cohort study, fTesto was chosen as the primary endpoint parameter for the RCT, due to its clinical, hormonal and metabolic impact and significance in PCOS pathophysiology and symptom severity. In addition, the other endpoints from the pilot trial would be adapted into the RCT as well.
Thus, secondary endpoints (with the testing method in brackets) would consist of:

- Glucose metabolism (Oral glucose tolerance test), including HOMA-IR, Matsuda indices, insulin and glucose AUC

- Other hormonal parameters of PCOS (AMH; ASD; FSH; LH; DHEA-S; 17OHP; 17-OH-estradiole; dihydrotestosterone, TT, 25OHD)
- Hirsutism (mFG-score)
- Body weight (BMI; waist-to-hip-ratio)
- Gut permeability and inflammation (Functional sugar test; surrogate parameters: serum DAO; stool zonulin; calprotectin; LPS-binding protein LBP; soluble cluster of differentiation sCD14; bacterial DNA)
- Gut lumen and mucosa microbiome composition and metagenomic profile (16S-RNA gene sequencing)
- Phytoestrogen production (soy challenge test; urine daidzein and equol concentrations)
- Quality of life (PCOS questionnaire; depression questionnaires; diet questionnaires)

In additional, exploratory endpoints were defined as:

- Lipid metabolism (LDL; HDL; LP(a); triacylglycerol;)
- FACS analysis (B cell subtypes)
- Metabolomics of stool and blood
- Gene expression analysis in blood and biopsy samples
- Changes in incretin levels (glucagon-like peptide 1 GLP1, glucagon, ghrelin, gastric inhibitory peptide GIP, peptide YY PYY

3.6.3 Treatment arms and allocation

The trial framework would serve to successfully fulfill the primary aim of proving probiotic intervention as a viable treatment option for PCOS. Therefore, a probiotic and a probiotic placebo treatment arm needed to be included in the trial design, with an equal number of participants in each treatment arm.

In order to achieve the secondary aim of a probiotic versus metformin comparison, an additional treatment arm with metformin therapy needed to be included in the trial, with a comparable number of participants as in the probiotic treatment arm. However, in contrast to the probiotic and probiotic placebo arms, the metformin arm was included as an open-label arm, as the metformin pills could not be disguised as probiotics or placebo, which were contained in sachets. In addition, the

efficacy of metformin has been proved over the decades times and times again, making a blinding of metformin obsolete and unnecessary for the purpose of this study.

In order to accommodate the dual nature of the trial and the inclusion of the third open-label metformin arm, two randomizations needed to be performed at the first visit.

The first randomization separated the double-blinded probiotic and placebo participants from the open-label metformin participants via a 2:1 ratio in an open manner. This was achieved with the Randomizer web tool (www.randomizer.at) developed at the Institute for Medical Informatics, Statistics and Documentation at the Medical University of Graz.

The double-blinded treatment arm participants underwent a second (blinded) randomization with a 1:1 ratio. This randomization was performed by the probiotic and probiotic placebo manufacturer Winclove Probiotics B.V., Netherlands. Both randomizations were stratified for endoscopy participation (yes/no) as described below, ensuring a level distribution of endoscopy volunteers across all three treatment arms.

3.6.4 Study schedule and visits

The timeline of the study for each participant is shown in figure 10, while the design is shown in figure 11.

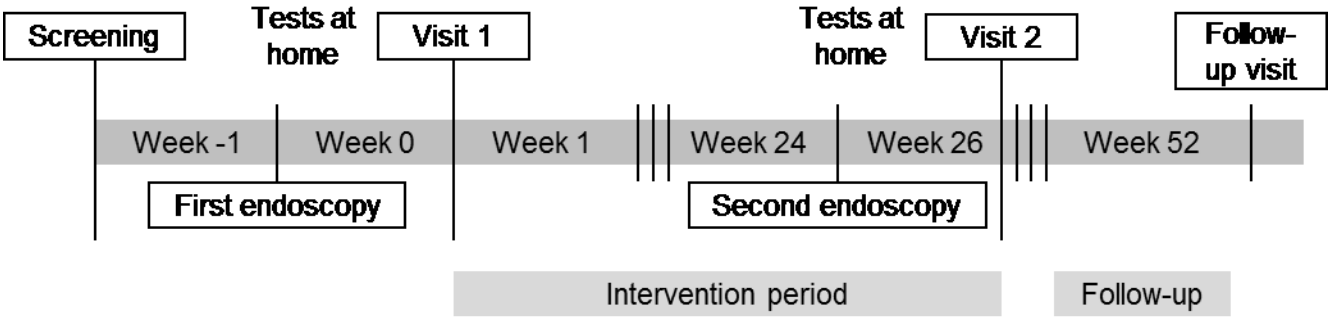


Figure 10: Participant visit schedule

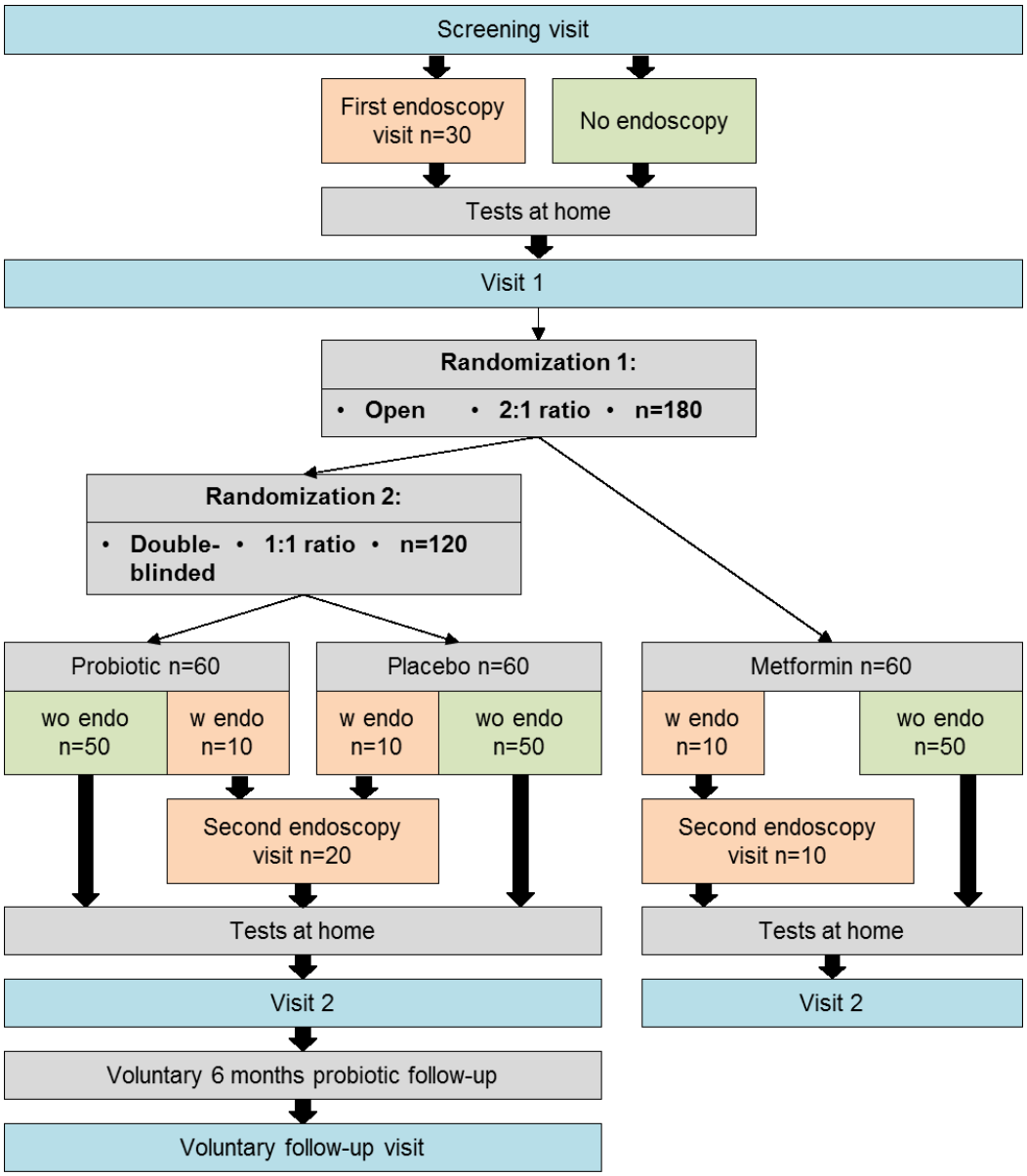


Figure 11: Trial Design of the ProPCO RCT trial; cited from (130)

All work related to the RCT would be conducted at the outpatient clinic of the Division of Endocrinology and Diabetology of the Medical University of Graz, Austria.

However, in contrast to the ProPCO Pilot trial, several key changes would have to be made to the visit schedule and to the tests performed:

First, in order to obtain intestinal wall biopsy samples for microbiome composition and metagenomics analysis, two endoscopic visits including a gastroscopy and recto-sigmoidoscopy would need to be scheduled between the screening and first visit as well as shortly prior to the

second visit. As this part of the study included an invasive procedure with additional possible complications and ramifications, it would need to be strictly voluntary and only done in a subset of participants from each treatment arm, since it would otherwise be impossible to recruit the necessary number of participants for the trial as a whole. Therefore only ten participants from each treatment arm would undergo these endoscopies, which would need to be scheduled separately from the study visits as these would take place at the endoscopy outpatient clinic of the Division of Gastroenterology and Hepatology of the Medical University of Graz. In order to compensate the volunteers for the additional time and risks involved with the procedures, they would be paid 200 euros each upon completion of both endoscopy visits.

Second, the soy challenge test would be extended for an additional day in contrast to the ProPCO Pilot trial, in order to increase the probability of detecting equol in the urine samples after soy milk consumption. Therefore, the tests at home would take three days at the RCT trial instead of two at the pilot trial.

Third, the trial duration would be doubled from twelve weeks to 24 weeks, in order to increase the probability of detecting beneficial effects from the intervention at the second visit with regards to the study endpoints.

Fourth, in order to potentially improve the health of the women who randomly participated by taking a probiotic placebo during the trial, a voluntary follow-up period would also be added after the second visit. This follow-up would be available to all women taking either a probiotic or probiotic placebo, and would consist of additional six months of intervention. However, all participants would only receive the probiotic product, no placebo would be included. This decision would be made in order to offer women who had taken a placebo previously to experience the potential benefits of the probiotic product, as well as monitor the effects of the probiotic after an additional six months in women who would have taken the probiotic previously, thereby doubling their intervention period.

As a result of scheduling difficulties during the pilot trial, the fifth measure would consist of a telephone visit at half-time during the intervention period in the RCT in order to more frequently communicate with the participants and to answer any questions they might have during the trial. The date of the second visit would also be confirmed during the telephone call in order to minimize potential misunderstandings and miscommunication (e.g. if a date was entered falsely in a calendar).

Screening Visit

Women with suspected or already established PCOS diagnoses would be approached and the trial and its goals would be explained by an investigator of the trial. Potential participants would each receive two informed consent forms (IC), one for the entire RCT, and one for the voluntary endoscopic procedures, as the latter required both additional information and were only available to a subgroup of participants.

Once any open questions were addressed and the main IC (and possibly the additional IC) signed, blood would be drawn for the evaluation of the inclusion and exclusion criteria, the study endpoint determinations and a safety lab, including a differential blood count, liver and kidney parameters, electrolytes, total cholesterol, LDL, HDL, triglycerides, Lp(a), and C-reactive protein (CRP).

In addition, participants would fill out quality-of-life-, PCOS-symptoms and diet-related questionnaires, a physical examination including blood pressure, body height and weight, Waist and hip circumference measurements and the measurement of the mFG-score would be performed.

Participants would finally be handed all utensils necessary for the isoflavone challenge and gut permeability tests including oral and written instruction on how to use them. The first study visit would then be scheduled to take place on the day after the last day of testing, as the gut permeability urine samples would need to be returned to the outpatient clinic at maximum 24 hours after collection.

First endoscopy visit

Prior to the tests at home, 30 volunteers would submit to a gastroscopy and recto-sigmoidoscopy at the Division of Gastroenterology and Hepatology, University Hospital of Graz. Recto-sigmoidoscopy was chosen as the preferred method of obtaining lower gastrointestinal biopsy samples in contrast to a full colonoscopy as it does not require a bowel emptying protocol, leading to several benefits for the trial conduct. In addition to improving patient adherence and willingness to participate in the procedure, circumventing bowel-emptying measures also decreases the chance of losing important microbiota in stool samples collected at home within a few days after the endoscopy. Furthermore, it negates the need for further patient preparation, study materials and possible confounding factors during the procedure itself in case the emptying was insufficient.

In total, 44 biopsy samples would be collected during the endoscopy visit: two routine gastric biopsies in the antrum and fourteen biopsies each from the gastric corpus, the pars descendens duodeni and the sigmoid colon 20 cm ab ano. From each site, two samples would be analysed via 16S-amplicon sequencing with regards to microbiome composition using Illumina technology

and the QIIME 2 pipeline. Two samples (including the two routine samples from the antrum) would be assessed using histological staining looking for morphological abnormalities and pathologies. Two samples would be used for gene expression analysis of transport proteins. These two samples from each region would be stored an RNAlater solution immediately upon extraction and stored at -20°C until analysis. RNA isolation would be performed using the RNeasy Mini Kit from QIAGEN GmbH, Austria. The remaining eight samples of each region would be used for FISH immunostaining of mucosal tissue and bacteria. Following several hours of observation at the endoscopy unit, study participants would then be discharged.

Tests at home

Undertaking the two tests at home would receive six urine tubes, three urine collection cups, one urine bottle, one liter of vanilla soy milk, two stool sample tubes and collection kits, two intransparent stool sample storage tubes, one urine extraction needle, written instructions for both tests, contact information of one of the investigators in case any questions or problems arose during the conduct of the tests, the sugar solution containing sucrose, mannitol and lactulose as well as the set date of the first study visit (and possibly the first endoscopic visit).

The first stool sample should be collected prior to onset of the isoflavone challenge test and frozen at -18° Celsius using an at-home freezer. For the test itself, 250 ml of soy milk should be ingested once in the morning and once in the evening for two consecutive days. After each day of soy milk ingestion, two overnight urine samples should be collected using the two urine cups (labeled A and B for day 1 and 2 respectively) and four urine tubes (labeled “day 1” and “day 2” respectively). All urine samples from the isoflavone challenge test should be stored in the freezer next to the first stool sample. Lastly, any stool after the last ingestion of soy milk should be collected using the second stool collection kit and sample tube and stored in the freezer as well.

After collecting the urine using the cup B after two days of soy milk consumption, a third urine tube labeled “Tube B” should be collected as well. This commences the gut permeability test. It is important that participants remain fasting during this test so as not to distort the sugar measurements from the three sugars in the sugar solution!

Afterward, participants should drink the sugar solution with at least 500 ml of water and collect any urine they produce for a period of five hours in the urine collection bottle (labeled “Bottle C”. The collected urine should be translocated into the last urine tube (labeled “Tube C”). Tube B and Tube C should be stored at +4° to +8° Celsius, presumably in a fridge , until the first study visit on the next day, since the samples should not be stored for longer than 24 hours at home.

First study visit

The following day, the participants would return with the collected urine and stool samples to the outpatient clinic. The results from the screening visit including the bloodwork would be discussed, and the participant would get randomized. Using the Randomizer tool from the Institute of Medical Informatics, Statistics and Documentation available at www.randomizer.at, participants would be randomized openly to metformin or probiotic/placebo via a 1:2 ratio. This randomization would also be stratified for the 30 volunteers undergoing endoscopic procedures, ensuring that ten participants would be assigned to each treatment arm.

While the metformin-assigned participants would receive the pills and instructions on how to take them, the participants in the probiotic/placebo arms would receive double-blinded numbered boxes containing either placebo or the probiotic.

After receiving the necessary materials for the repeat of at-home testing and scheduling the second study visit (and second endoscopy visit where applicable), participants would be sent home to begin their 180-day intervention period.

Telephone Visit

After approximately three months of intervention, participants would be contacted via phone call to assess their well-being, their adherence to the study protocol and to confirm the date of the second study visit. If a participant could not be reached, three attempts should be made in total, including email contact before declaring a failed attempt of contact.

Second study visit

At the end of the intervention period, participants would repeat the tests at home as described above, before appearing for the second study visit. The tests and questionnaires from the screening visit would be repeated, including the oral glucose tolerance test.

Participants taking either the probiotic or placebo would then be offered the opportunity to participate in a follow-up period of another 180 days, during which they would take the probiotic product from the trial, albeit in an open label form. This would enable the investigators to examine a prolonged exposure to the probiotic in those participants who had been randomly assigned to the probiotic arm, as well as the effects of the probiotic on the participants from the placebo group in a cross-over fashion. Since this would only be a voluntary follow-up phase of the trial, no placebo was included, as there was no way to be sure how many participants would want to continue on,

making sample size and power calculations moot. In addition, this approach was chosen as a way for participants from the placebo group to also experience the effects of probiotic intervention, without again risking being assigned to a placebo treatment.
Patients would also receive a clinic report including their routine blood work with recommendations from trial investigators on additional measures they might explore to mitigate their PCOS symptoms. They would then be discharged.

Follow-up visit

The follow-up visit would be a repeat of the second study visit, with the exception of no tests being performed at home prior to their appearance at the outpatient clinic. This approach was chosen in order to improve patient comfort during this time and ease the scheduling of the follow-up study visit.

A summary of the trial procedures and visit schedules is provided in table 7, while a summary of the trial parameters and collected participants´ specimens is provided in table 8.

Visits	**Screening visit**	**Endoscopy 1**	**Visit 1**	**Endoscopy 2**	**Visit 2 End of Intervention**	**Voluntary follow-up**
Weeks +/- 3 or more days	(-4)-(-1)	-1	0	24-26	26	52
Patient history						
Extensive patient medical history	x					
Changes in medical history			x		x	x
PCOS history and symptoms	x				x	x
Randomization			x			
Endoscopic procedure		x		x		
Examinations						
Body weight/height	x				x	x
Waist/Hip circumference	x				x	x
Blood pressure, heart rate	x				x	x
mFG score	x				x	x
Questionnaires						
Diet (General, vitamin D and calcium)	x				x	x
PCOS	x				x	x
Depression (BDI, ADS)	x				x	x
Bristol Stool Scale	x				x	x
Laboratory						
Hormone panel	x				x	x
Oral glucose tolerance test	x				x	x
Safety lab (not an endpoint)	x				x	x
Coagulation safety lab	**x**					
FACS	**x**				**x**	**x**
P800 and PAX gene tube	x				x	x
Inflammation, gut permeability	x				x	x
Urine tests	x				x	x
Pregnancy tests	x				x	x
Isoflavone intake test	Between first and screening visit			Before the second visit		
Gut permeability test	After isoflavone intake test			After isoflavone intake test		

Table 7: Overview of study procedures and visit schedules

Measurement	Material and volume (per visit)	Visit	Study endpoint	Routine/Study specific	Laboratory
PCOS-related hormones and surrogate gut permeability markers: total and free testosterone, SHBG, AMH, androstenedione, DHEA-S, 17-hydroxy-progesterone, 17-hydroxy-estradiole, LH, FSH, fT3, fT4, TSH, TPOAK, TGAK, lactase genetic analysis, DAO, zonulin, sCD14, LBP **Endocrine safety lab** Cortisol, ACTH, prolactin, IGF-1	3 x 8 ml serum 1 x 3 ml EDTA 1 x 3 ml EDTA 1 x 8 ml urine	Screening Visit 2 Follow-up Visit	Yes (except safety lab)	Routine Study specific: DAO, zonulin, sCD14, LBP	Endocrinology Lab Platform, Division of Endocrinology and Diabetology, Medical University of Graz
Oral glucose tolerance test: Insulin, glucose and C peptide levels at baseline as well as 30, 60 and 120 minutes after ingestion of 75 mg of glucose solution	4 x 3 ml serum 4 x 2 ml sodium flouride	Screening Visit 2 Follow-up Visit	Yes	Routine	Endocrinology Lab Platform
Metabolic parameters: GLP1, glucagon, ghrelin, GIP, PYY in blood	1 x 2 ml P800 tube	Screening Visit 2 Follow-up visit	Yes	Study specific	Endocrinology Lab Platform
Gene expression: Glucose metabolism pathways Isoflavone metabolism pathways Androgen metabolism pathways	1 x 2.5 ml PAXgene tube	Screening Visit 2 Follow-up visit	Yes	Study specific	EndoGeneLab, Division of Endocrinology and Diabetology, Medical University of Graz
B-cell FACS	2 x 8 ml heparin tubes 1 x 3 ml EDTA	Screening Visit 2 Follow-up visit	Yes	Study specific	Immunology Laboratory, Department of Internal Medicine, Division of

					Rheumatology and Immunology, Medical University of Graz
Safety lab, lipid profile and inflammation: Differential blood count, liver and kidney parameters, electrolytes, total cholesterol, LDL, HDL, triglycerides, Lp(a), hsCRP	1 x 3 ml EDTA 1 x 8 ml heparin tube 1 x 8 ml serum	Screening Visit 2 Follow-up visit	Only cholesterol LDL, HDL Triglyceride, Lp(a), hsCRP	Routine Study specific: Lp(a)	Clinical Institute of Medical and Chemical Laboratory Diagnostics (KIMCL), Medical University of Graz
Coagulation safety lab Prothrombin time, activated partial thromboplastin time	1x4,5 ml sodium citrate tube	Screening	No	Study specific	KIMCL
Biobank samples	1 x 8 ml serum 1 x 6 ml EDTA 1 x 8 ml urine	Screening Visit 2 Follow-up visit	No	Study specific	Biobank Graz, Medical University of Graz
Functional gut permeability test: Sucrose, lactulose, mannitol	2 x 8 ml urine	Visit 1 Visit 2	Yes	Study specific	Gottfried Schatz Research Center for Cell Signalling, Metabolism and Ageing, Molecular Biology and Biochemistry, Laboratory of Integrated Structural Biology & Metabolism Research, Medical University of Graz
Isoflavones: Daidzein, Equol	4 x 8 ml urine	Visit 1 Visit 2	Yes	Study specific	Endocrinology Lab Platform
Microbiome Analysis and gut inflammation: 16S rRNA gene sequencing calprotectin, zonulin	2 x 10 ml stool samples	Visit 1 Visit 2	Yes	Study specific	ZMF Core Facilities, Graz Endocrinology Lab Platform (Zonulin, Calprotectin)

Biopsy samples: Histology, FISH immunostaining, 16S rRNA gene sequencing, gene expression of isoflavone transporters	44 endoscopic biopsy samples	Endoscopy 1 Endoscopy 2	Yes	Study specific	To be determined*
Total volume per visit	**Blood: 92.5 ml (97 at the screening visit); Urine: 64 ml; Stool: 20 ml; Endoscopy: 44 biopsies per visit**				

Table 8: Participant specimen and their corresponding (endpoint) parameters; *Histological samples would be analyzed at the Diagnostic and Research Institute of Pathology, Medical University of Graz, while the locations of the other analyses have not been finalized as of July 2024

3.6.5 Inclusion and exclusion criteria

Similarly to the ProPCO Pilot study, premenopausal women aged between 18 and 45 meeting at least two of the three Rotterdam criteria who sign the informed consent form for the trial would be eligible to participate, provided they did not meet any exclusion criteria.

These were also modelled after the criteria used in the ProPCO Pilot trial, with several modifications due to additional concerns for concomitant therapies, which could potentially alter the gut microbiome:

The entirety of the exclusion criteria for the RCT would thus include missing or withdrawn consent, other causes of HA, such as Cushing´s disease, hyperprolactinemia, adrenal conditions or tumors or previously diagnosed genetic disorders with association of hyperandrogenism.

Pregnant or nursing women within the first six months after the birth of their child/children were also excluded, as were women with allergies or food intolerances pertaining to the intervention product or trial procedures, such as soy allergy or lactose intolerance.

Due to their potentially altered metabolism, anatomy or changed gut microbiome compositions, patients with type 1 diabetes, chronic inflammatory bowel disease, history of gastrointestinal cancers or major surgeries in the gastrointestinal tract with the exception of appendectomies or cholecystectomies, acute gastrointestinal infections, any malignancies requiring treatment within the last three years prior to recruitment, chronic illnesses requiring regular checkups or hospital treatments at least once every three months with the exception of type 2 diabetes (which is a known complication of PCOS) as well as patients with known drug or alcohol abuse history were also excluded from trial participation.

Furthermore, women taking antidiabetic drugs, proton pump inhibitors, hormonal contraceptives or other oral steroid derivatives within six months prior to recruitment and/or antibiotic treatment

up to three months prior to recruitment were classified as screening failures as well, due to the drugs´ potential to alter the subjects gut microbiome or PCOS metabolism (117,131). In those cases, women were invited to get rescreened at a later point in time, when they would no longer be excluded under these time-sensitive premises.

In addition to the medications precluding a woman from participating in the trial, other medications would be expressly allowed as concomitant therapies, such as thyroid hormone derivatives, angiotensin receptor antagonists and angiotensin-converting-enzyme antagonists (ACE-, AT1-inhibitors). Many other medications, herbal and homeopathic remedies are not mentioned or discussed in the protocol and would be evaluated at the screening visit based on their effect on PCOS metabolism or gut microbiome composition as defined by the exclusion criteria.

3.6.6 Intervention product

Based on lessons learned from the pilot trial and the planned design of the study, the commercially available OMNi-BiOTiC® metabolic probiotic product contained corn starch, maltodextrin, fructo-oligosaccharides, galacto-oligosaccharides, polydextrose, plant proteins, potassium chloride, magnesium sulfate, the aforementioned seven different bacterial strains and manganese sulfate. This product will be altered as follows:

Instead of 3 billion colony-forming units, one dose of probiotic sachet would contain 6 billion colony-forming units in order to increase the potential benefits, the rate of colonization of the gut mucosa and possible detection in stool samples. Additionally, 2000 IU of vitamin D (25OHD) would be included as a prebiotic.

The probiotic placebo will look, smell, taste and feel identical to the placebo and contain most of the ingredients of the probiotic product as well. It will contain corn starch, maltodextrin, potassium chloride, magnesium sulphate and manganese sulphate.

Both products will be produced by Winclove Probiotics B.V., Netherlands, as part of a mutual collaboration within the K1 centre CBmed project 3.22, as outlined above.

The metformin used in the RCT will be commercially available Glucophage 500 mg, manufactured by Merck Santé S.A.S., Semoy, France and distributed by Merck GmbH, Vienna, Austria. The product will be acquired through the local pharmacy Apotheke St. Leonhard Mag pharm Brettner KG, Graz, Austria. Merck or its subsidiaries are not involved nor do they profit (additionally to the regular market price of their product) from this RCT.

The probiotic and placebo groups received 4 boxes of product containing 200 sachets in total, to be used for up to 180 days of intervention once daily, followed by the second study visit. The number of unused sachets was counted at that time to determine the number of days the product was consumed, with a discrepancy of ± five days being allowed by the study protocol. If the discrepancy were higher, the participants would be analysed in the intention-to-treat analysis, but not in the per-protocol analysis. Each box had all the ingredients of the probiotic sachets written down, with a "±" added to the ingredients not present in the placebo.
Similar conditions would apply for the metformin users, though they would receive 2 boxes with 400 pills of metformin in total, to be used twice daily for the same amount of time. At the end, the number of pills would be counted and compared to the expected number of remaining pills, had the participant taken all pills as required. As the intake frequency would be double the number of probiotics/placebos taken, a discrepancy of ± ten pills was allowed at the second visit for the data to be included in the per-protocol analysis as well instead of only the intention-to-treat analysis. As the metformin arm was not blinded, no special packaging was provided and all information regarding product safety and its use was included in the commercially available product packaging.

3.6.7 Statistical analysis

Disclaimer: The statistical analysis of the RCT will be performed by Dr. Regina Riedl from the Institute of Medical Informatics, Statistics and Documentation, Medical University of Graz. The statistical analysis plan for the RCT was also designed primarily by her. This section is included in this thesis to show the impact of the two preparatory studies on the statistical analysis, and is not a part of the applicant´s original work. As such, this section does not contain the entire statistical analysis plan, but only relevant excerpts.

The design of the study aims to primarily compare the effects of six months of probiotic intervention with a placebo, in order to show that probiotic treatment is superior to the placebo. As a secondary consideration, the probiotic treatment arm is compared to the metformin arm to show non-inferiority.
Based on the results from the pilot trial and the choice of fTesto as the primary endpoint parameter, the sample size calculation was conducted as described above. The changes in fTesto levels between the two groups will be compared using analysis of covariance (ANCOVA), with fTesto levels at 6 months defined as the dependent variable and fTesto at baseline as well as the

treatment group allocation defined as covariates. If the assumption of a covariance is not fulfilled, the changes in fTesto levels between the second and screening visits will be compared via t-test or Mann-Whitney-U-test, with a two-sided p-value of less than 0.05 being considered statistically significant.

The secondary endpoints would be analysed in a similar manner as described above using parametric or non-parametric tests as appropriate. All statistical analyses will be conducted using the SAS program, version 9.4, SAS Institute, North Carolina, USA.

The microbiome will be analysed separately by the investigators of the trial directly, using the Galaxy server of the Medical University of Graz containing the QIIME 2 pipeline.

The data for these analyses will be derived from the case-report form in the case of biometric measurements and patient history, from the respective laboratories in the case of blood parameters, or from the questionnaires the patients fill out.

3.6.8 Safety protocols

Several precautions were implemented for the RCT in order to minimize potential risk factors for our participants.

A thorough patient history was taken at the beginning of the screening. Our exclusion criteria were defined very broadly, not only to reduce potential sources of bias, but also to prevent patients with a higher risk of adverse events from our study to be included.

Probiotics and also metformin are considered generally safe to use and the expected number of serious adverse events was negligible to none due to the study interventions products.

Due to the endoscopy visits with additional risks, any known risk factors were considered exclusion criteria. In the case of unknown conditions or risks which were only discovered during the first endoscopy visit, our protocol dictated that the procedure should be discontinued immediately.

All patients were monitored for several hours after the procedure and were required to be under observation by a relative or friend during the rest of the day.

In the case of any (suspected) adverse events during the intervention period, participants were required to notify the investigators immediately via an emergency contact number they received with their informed consent forms. Any (suspected) serious adverse events would have had to be immediately forwarded to the institutional review board as well as the Austrian authorities, as required by Austrian law.

4 Discussion

4.1 Probiotic intervention in PCOS – Comparison with prior literature

Both the pilot trial and the PCOS cohort study served their purpose and demonstrate the feasibility and potential benefit of a large-scale probiotic RCT. By providing answers to all relevant procedural questions required for the RCT, they laid the groundwork for designing a triple-arm study comparing a probiotic to a placebo as well as metformin as a standard treatment, the first trial of its kind to do so.

Mounting evidence from prior RCTs suggests the potential of using probiotic supplements for the treatment of PCOS, however lack of standardization in the methodology of these studies requires a more in-depth comparison to highlight the novelty of our planned RCT compared to previous studies.

4.1.1 Low sample sizes in previous publications

Most prior RCTs were conducted on 30 women or less per intervention arm (100–102,105–108,110,112), and even compared to the two studies that recruited more participants (103,111), our study has a planes sample size of 60 per treatment arm, exceeding these RCTs. Furthermore, while our sample size calculation is based on fTesto reduction shown in the pilot trial, based on the same methodology and clinical setting planned for the RCT, many of the aforementioned studies did not mention their rationale or basis for their respective sample sizes (101,102,110,112), or did not match their recruitment numbers for the trial (103). Another study (106) cited their sample size calculation based on published literature (132), however in the case of the latter study, participants consisted of pregnant women instead of women with PCOS, while using HOMA-IR as the primary outcome measure for the calculation in both cases.

Discrepancies such as the ones mentioned above are greatly detrimental for the purpose of reproducing the published results, and should be avoided if possible. In contrast, our sample size calculation uses a clearly defined primary outcome, based on the clinical significance for the patient collective, thereby enabling its reproducibility.

4.1.2 Probiotic compositions vary greatly and are inherently imprecise

Other metrics of methodology also varied greatly among the RCTs, such as the choice of probiotic products/strains, inclusion of prebiotics, inclusion and exclusion criteria for recruitment, outcome measures and areas of interest and the resulting significant results and conclusions.

Apart from one study mentioning diverging ingredients in the group description and intervention section (108), all studies mentioned above utilized a multi-strain probiotic mixture combined with prebiotic carbohydrates, sometimes in addition to further prebiotic additives, such as inulin (103,105,106,110), vitamin D (107) or selenium (101). Four studies from Iran used the same probiotic mixture, containing *Lactobacillus acidophilus*, *Lactobacillus casei* and *Bifidobacterium bifidum*, at a quantity of $2x10^9$ CFU/g each (100,102,105,106). In total, *Lactobacillus acidophilus* was used most commonly, with six more intervention products including it in their probiotic mixture (101,103,107,110–112).

In contrast, our pilot study compared three different probiotic products sold by Institut Allergosan, OMNi-BiOTiC® STRESS Repair, OMNi-BiOTiC® 6 and OMNi-BiOTiC® metabolic. All three products contained multiple probiotic species, including *L. acidophilus*, though every product used a different subspecies. STRESS Repair used *L. acidophilus* W22, 6 contained *L. acidophilus* W55, while metabolic included both *L. acidophilus* W22 and W37 subspecies. In most studies above the specific subspecies used in the probiotic products are not mentioned.

While the authors do not elucidate on the reason for including *L. acidophilus* in most of these studies, its properties make it a very desirable ingredient for commercial probiotics. These include a resistance to gastric and bile acids, a preference for anaerobic growth, while also tolerating oxygenated environments, as well as their versatility in utilizing glucose, fructose and lactose as substrates for fermentation (133).

In addition to their adaptable physiology, their metabolic products offer a variety of health benefits, with a degree of variation across different subspecies, including inhibition of cardiovascular disease as well as gastrointestinal disease progression including colon and hepatic cancers (133).

Lactobacillus casei was another probiotic species commonly used in the above mentioned RCTs (100,102,103,105,106,111,112), while its subspecies W56 was the only strain used in all three probiotic products in our pilot trial. This species is also commonly used in probiotics and shares many characteristics with *L. acidophilus*, as well as many of its health-promoting anti-inflammatory and anti-cancer metabolic effects (134,135). However there are many different subspecies, with a high degree of variance in environmental and metabolic properties, hampering a direct comparison between two or more studies using this species, especially if the strains are not specified further.

This problem is further exacerbated by a change in nomenclature in 2020 due to new phylogenetic discoveries. As a result, many lactic acid bacteria including the genus *Lactobacillus* received new designations and were assigned different taxonomies than prior to 2020 (136). As a result, studies conducted after 2020 are even more difficult to compare to prior ones due to the changes nomenclature status of many probiotic strains used. Since most of the studies assessing the effects of probiotics in PCOS were conducted and published prior to 2020, including our own pilot trial and the protocol design for the large RCT, this thesis chose to use the older terminologies in order to increase the comprehensibility and the possibility of tracking the authors thought processes when reading the aforementioned publications.

The other probiotic species used in OMNi-BiOTiC® are either unique to a particular product, or appear in metabolic and one other product, such as *L. acidophilus* W22 and *Lactobacillus plantarum* W62 being included in metabolic and STRESS Repair, as shown in figure 2. Of these, *Bifidobacterium bifidum* used in OMNi-BiOTiC® STRESS Repair is another very commonly used probiotic strain in previous RCTs investigating probiotic intervention in PCOS (100–102,105–107,111,112).

Similarly to *L. acidophilus* and *L. casei*, *B. bifidum* has been found to play an immune-modulatory and anti-inflammatory role in the gut, exerting a positive influence on a number of different conditions and diseases, such as inhibition of H. pylori, reduction of necrotizing enterocolitis in infants and improving lipid and inflammation markers as well as the gut barrier (137,138). Its latter properties may be especially useful for PCOS treatment, as many women exhibit both lipid and gastrointestinal dysregulation, leading to systemic inflammation and increased body weight (13,16). Interestingly, while many of the probiotics used previously could demonstrate a positive effect on lipid profiles, in our pilot study the group using OMNi-BiOTiC® STRESS Repair was the only intervention group showing an increase in BMI over time, instead of a decline, as it would be expected. However, this could be explained both by the low sample size, as well as the higher starting BMI in this group (mean of 33.1 kg/m² vs. 25.1 in the OMNi-BiOTiC® 6 group and 23.3 in the OMNi-BiOTiC® metabolic group). Despite this negative result in our pilot trial, future RCTs investigating probiotic intervention in PCOS should focus on probiotic strains showing a beneficial effect on lipid and/or glucose profiles, and should furthermore demonstrate that any relevant clinical improvement in these women is a direct result of the improved gut metabolome.

4.1.3 Variations in inclusion and exclusion criteria

General principles of inclusion and exclusion criteria in PCOS

Apart from the intervention itself, choosing adequate inclusion and exclusion criteria for the intervention study can have as big an impact on the results. This principle is especially crucial in probiotic intervention studies for the treatment of PCOS symptoms, since the disorder as well as the intervention have a multitude of metabolic interactions and pathways to consider, including prior medical history and the intake of additional medications.

In the case of PCOS, any known and accepted treatment of PCOS poses the risk of result bias, since any symptom improvement would be difficult to link to the intervention instead of the known treatment, such as COCs or metformin.

In contrast, prior history of type 1 diabetes mellitus for instance, not associated with PCOS, naturally impacts glucose metabolism metrics, as does the insulin therapy needed for survival in such patients.

Menopause is another important confounding factor to consider in women with PCOS, as menstrual cycles cease and thereby negate one of three defining traits of PCOS according to the Rotterdam criteria. In addition, changing hormone levels can greatly impact any results assessing changes in androgen levels after an intervention with probiotics.

For these reasons, it is common practice to impose an age limit in PCOS studies as well as exclude women taking standard PCOS medical treatment options, such as metformin and COCs. However, this in turn severely limits the potential pool of participants in any PCOS intervention trial, since most women diagnosed with PCOS suffer from its symptoms and wish them to be treated as soon as possible.

This leaves investigators with four choices if they wish to recruit women with PCOS for an intervention trial:

First, they can try to persuade women already taking some form of treatment to discontinue it, wait an appropriate time for their effects to wear off, and then recruit them for a potential novel treatment option which has not been proven to work. Needless to say, the number of expected successful recruitments using this method should be expected to be low.

Second, they can try to recruit women directly after they receive their PCOS diagnosis, prior to any treatment onset. This method offers the advantages of recruiting completely therapy-naïve women, reducing the risk of bias, while also providing the participants with additional blood tests, clinical observation and medical support during the trial. However, the possible window of

recruitment is very narrow, and the likelihood of recruiting younger women for the studies increases, which may present a source of bias.

Third, investigators could attempt to recruit women who, for whatever reason, discontinued their prior PCOS treatments, such as women ceasing COC treatments due to a thromboembolic event or their wish to get pregnant, or discontinuing their metformin treatments due to gastrointestinal side effects. While this method may increase the adherence of affected women who do choose to participate, it requires investigators to keep track of their PCOS patients over the years on a long-term basis, and almost necessitates a corresponding PCOS cohort study to be administered at the same location as the intervention trial. In addition, these women represent a minority among those affected by PCOS, meaning a prolonged period of recruitment, thereby delaying trial analysis and increasing the financial burden of the trial.

Fourth, investigators can accept a degree of result bias by recruiting women taking some form of PCOS treatment but not another, or women suffering from some additional disorders which might impact the results, but not others, which could be expected to have a severe impact on trial results.

In most cases, a combination of several of these options is used in order to increase the recruitment speed and try to minimize exposure to unnecessary biases.

Additional considerations in probiotic trials

While it is already difficult to recruit women for any PCOS intervention trial, probiotic intervention trials impose further restrictions on possible participants.

Since the area of effect of probiotics is the gastrointestinal tract, any prior history of severe gastrointestinal diseases, such as cancer, or inflammatory bowel disease, may have a severe impact on the results of probiotic ingestion. In addition, many common chronic diseases and disorders have been shown to impact microbial composition and diversity (see chapter 1.4).

Furthermore, while there are many medications and treatments affecting PCOS symptoms, even more may interact with the gut microbiome, due to oral consumption and the necessity of absorption via the gut. The most common medications associated with a severe impact on the gut microbiome are antibiotics (due to their very nature as antibacterial substances inhibiting their growth or killing them outright), as well as proton pump inhibitors (by reducing the gastric acidity and allowing microbes from the upper GI tract and mouth to enter the lower GI tract) (129,139).

Another drug impacting the microbiome significantly is metformin, by increasing the abundance of *Escherichia coli* and 80 other known microbiota and influencing short-chain fatty acid metabolism in the gut. In fact, the common gastrointestinal side effects of metformin may be explained by its

effects on the gut microbiome (140).The effects of these drugs on the microbiome have been reported to last several years after discontinuation, with conflicting results over several studies (117,131).

Other drugs known to impact microbiome composition and function are statins, opiods, ACE inhibitors, beta blockers, laxatives and antidepressants such as selective serotonin reuptake inhibitors (SSRI). While ACE inhibitors, opiods, statins and beta blockers are not as widespread among young women as they are in older subjects, SSRIs may be encountered as daily drugs, especially in young women with PCOS, who may experience signs of depression due to their symptoms, such as unfulfilled child wish, obesity and hirsutism. However clinicians should consider SSRI use in PCOS carefully, in particular due to the possible additional weight gain that can accompany the use of such drugs (141).

Nevertheless, all of these drugs need to be considered when designing a probiotic intervention trial in PCOS. However, if one were to exclude prior use of any microbiome-altering drug, the recruitment process could be expected to grind to a halt. Therefore, a careful balance between excluding the most egregious microbiome-affecting drugs (over a suitable and clinically practical time frame prior to recruitment) and finding a suitable number of women with PCOS able and willing to participate needs to be implemented in such cases.

Comparison of inclusion criteria in probiotic intervention RCTs

While all studies mentioned above used the Rotterdam criteria for PCOS diagnosis confirmation (100,101,110–112,102–109) there was great variation in weight ranges and ages as well as other criteria in the inclusion criteria.

Most studies conducted in Iran chose an age range of 18 to 40 years (100–102,105–107), as did Kaur et al. in India (111), while Karimi et al. recruited up to the age of 37 instead of 40 (103), Esmaeilinezhad et al. recruited from 15 to 48 years of age (108) and the other studies did not specify an age limit.

More differences were found in weight criteria for inclusion, from BMI ≥ 19 kg/m² (100), BMI ≥ 25 kg/m² (103,110) and a BMI range of 17-34 kg/m² (107), while most studies did not specify weight restrictions when considering inclusion criteria.

Furthermore, Ahmadi et al. only included phenotypes A (HA+OM+PCOM) and D (OM, PCOM) in the trial, without elucidating on the reason for excluding the other two phenotypes of PCOS (100). Contrarily, while we also defined PCOS via the Rotterdam criteria and further defined clinical and biochemical hyperandrogenism, our age limit extended to 45 years of age, and we did not assess

PCOM presence at our outpatient clinic directly, as we had no gynecologists or transvaginal ultrasound capabilities present at our study cite.
In contrast to most other studies referring to the Rotterdam criteria, based on a more recent guideline, we defined hirsutism as an mFG score of ≥ 4 points (118). However, most studies cited above were conducted on Iranian women with PCOS, where other mFG cutoffs may be applied due to different ethnicities and affinity for facial hair development (118).

Comparison of exclusion criteria

While the inclusion criteria showed some variety centered on the Rotterdam criteria, the exclusion criteria diverge even more among the above mentioned studies.
Ahmadi et al. excluded women with type 2 diabetes mellitus, active liver disease, cardiac or renal failure, hormone medication, thyroid disease, adrenal hyperplasia, antiobesity or antidepressant therapies within three months of enrollment, smoking, as well as consumption of antibiotics and fermented foods including kefir and yogurt (100). In contrast, PPI and metformin therapies were not mentioned, though “subjects were requested […] not to take any medications that might affect findings during the 12-week intervention” (without specifications), nor did the authors mention specific criteria for some of the conditions involved. This applies to the term “active liver disease”, which can range from metabolic dysfunction-associated steatotic liver disease (MAFLD), formerly known as non-alcoholic fatty liver disease (NAFLD), to liver cirrhosis and/or cancer. While the latter can be considered rare and late-stage expressions of various liver diseases, the former occurs in up to 55 percent of women with PCOS (142). Clarity is missing both in the definition of “active liver disease” as well as any specific diagnostic markers that were evaluated for said diseases. The same is true of the term “hormone medication”, which can range from hormone-impacting drugs, such as ACE inhibitors and beta blockers, as well as hormonally active drugs, such as COCs, insulin, cortisol etc. Without specifics, the study and its results cannot be properly examined or repeated, thereby slowing the progress of evidence-based treatments in this area.
Jamilian et al. only mentioned PCOS-specific exclusion criteria, such as adrenal hyperplasia, androgen-secreting tumors, hyperprolactinemia, thyroid dysfunction, diabetes at the time of enrollment or pregnancy (101). No mention of probiotic, antibiotic, PPI or metformin use was included, nor were any other conditions which might influence or decrease the microbial diversity.
Karamali et al. used simplified and shortened exlusion criteria, similar to Ahmadi et al., and excluded smokers, probiotic users (with no specification for dietary supplements and/or fermented

foods), “endocrine diseases including thyroid, diabetes, and/or impaired glucose tolerance”, as well as pregnant women. Use of antibiotics, PPI or metformin was not mentioned (102).

In contrast, Karimi et al. excluded any women with prior history of chronic heart, lung, cardiovascular, kidney or liver disease as well as autoimmune and thyroid disorders, known allergic reactions to one or more ingredients of the intervention product, use of antibiotics within three months prior to enrollment, and finally “multivitamin mineral supplements, and certain diet or physical activity programs” (103). These criteria leave room for serious bias with regards to the study results, as any women taking standard treatments for PCOS, such as COCs or metformin, could be included in the trial and impact the results. Similar issues with the exclusion criteria can be found in most other studies as well (105–107,112). Three other studies mentioned hormonal therapies in addition to antibiotics as exclusion criteria (108,110,111), however only Kaur et al. mentioned antidiabetic therapies explicitly (111). While many of the studies above mentioned type 1 and/or 2 diabetes in the exclusion criteria, they discount women with PCOS taking metformin not to treat diabetes (which they may or may not have), but to improve their PCOS symptoms. However, this again is a serious source of bias, as metformin impacts both hormonal levels and microbial composition of the host (see chapter 1). PPIs were not mentioned in any of the cited trials, though some studies excluded gastrointestinal disorders, presumably (as no specifics are given) including gastritis or reflux disease, the two most common indications for PPIs (105,108).

In contrast, we excluded women with other potential causes of HA and/or OM, such as CAH or hyperprolactinemia, while also excluding women who had taken metformin, oral contraceptives and/or antibiotics within a specified time frame prior to trial recruitment. We specifically only excluded type 1 diabetes mellitus, as we considered type 2 diabetes to be a sequela of PCOS. We did not mention liver diseases as well, partly for the same reason as diabetes, and partly to assess whether women with known liver diseases could represent a subgroup within the larger RCT, with the option of refining the exclusion criteria further later on.

This is precisely what happened during the RCT design, with more stringent timeline restrictions for standard PCOS therapies and antibiotic use were implemented (six months prior to enrollment in the trial, for the sake of simplicity and to minimize any impact of said medications in patients who had taken them previously and possibly discontinued using them prior to their trial participation.

In addition, we added IBD, GI tract surgeries (except appendectomies and cholecystectomies) and cancers, as well as the use of PPIs and/or systemic steroids within 6 months prior to recruitment, in order to reduce potential bias due to altered anatomy and/or microbial composition

due to these factors. For the same reasons, any chronic conditions unrelated to PCOS requiring check-ups and or treatments more frequently than once every three months, were also excluded, as were any malignancies that were treated within three years prior to the screening visit of the patient.

Other medications were expressly allowed during the trial, such as thyroid hormones and antihypertensive medications, as their underlying conditions were considered potential complications of PCOS (see chapter 1). In the case of thyroid dysfunction or adenomas, such as Graves´ disease, any active disease would require more regular checkups than every three months, thus being excluded a priori by the above criterion, negating the need to mention it somewhere else in the exclusion criteria.

Our goal was to ensure reproducible and sensible exclusion criteria, enabling recruitment of PCOS women possibly already suffering from metabolic sequelae, while reducing other confounders which could falsify our results.

4.1.4 Incomplete endpoint collection as a source of bias

Other criteria to evaluate the quality of an RCT is by examining the explored endpoints, both primary and secondary. While the primary endpoint is the most important outcome measure to assess the main research question of the study, the secondary endpoints add further data points to reinforce the results of the trial (143). Therefore it is important to select proper endpoints for the research question beforehand, and to consider the disorder, the research question as well as the intervention in the trial accordingly.

For an intervention trial involving PCOS, key features of PCOS should always be assessed to determine the clinical use of the intervention. This includes the diagnostic criteria for PCOS, such as cycle length and signs/biomarkers of HA. In addition, known metabolic complications, such as IR, diabetes and hyperlipidemia should also be considered through surrogate parameters or biomarkers (e.g. HOMA-IR, glucose levels and glucose AUC, HDL, LDL, triglycerides etc.). To negate one or more of these areas is to negate the clinical reality of these patients and to negate parts of the disorder one wishes to treat. Therefore, each of these areas should be addressed by at least one endpoint respectively, more if possible, independent of the choice of the primary endpoint.

Furthermore, when the intervention trial is attempting to improve PCOS symptoms via microbiome modulation (i.e. probiotics), further endpoints should be evaluated as a quality control measure.

While the probiotic strains themselves may or may not be detected through microbiome analysis, e.g. stool sample collection and 16S-RNA sequencing, alpha diversity metrics can be assessed as surrogate parameters for the expected improvement of the gut microbiome function, even if more extensive (and expensive) methods are not available. However, even 16S sequencing from stool samples can be time- and resource-consuming, and smaller self-financed intervention trials might lack the means to fund it. In such a case, this limitation should clearly be addressed by the authors in their discussion.

In the case of the aforementioned publications, most focused either on glucose and lipid metabolism (100,103,106), or on hormone levels, associated PCOS endpoints, depression endpoints and markers for oxidative stress (101,102,105,107,112), with virtually no overlap in between in the examples above. Only three of the studies included both areas of interest (108,110,111), while none of the studies above collected stool samples for microbiome analysis, leaving a serious gap in knowledge regarding probiotic intervention in PCOS.

In contrast, not only do our pilot and the proposed RCT study assess both PCOS criteria and metabolic sequelae, but we also collected stool samples for microbiome analysis, and in the RCT plan to compare alpha and beta diversity metrics across all groups, filling the gap mentioned above.

In summary, the proposed RCT is a well-designed intervention trial, comparing probiotic intervention not only to a placebo, but also with metformin, a key microbiome-altering antidiabetic drug used as a standard treatment method in PCOS.

Furthermore, to our knowledge, this will be the first probiotic intervention trials in PCOS that will obtain microbiome data through stool collection as well as mucosal samples in a subgroup of participants, enabling the comparison of lumen versus mucosa colonization of microbiota, another topic of interest in PCOS with no prior data as far as we are aware. If successful, new treatment approaches could be offered women who suffer tremendously under such a common endocrine, metabolic (and microbial) disorder, which makes it of paramount importance.

4.2. PCOS phenotypes and beyond

In addition to facilitating the design of a new probiotic intervention trial in PCOS, the cohort study results demonstrate the difference between the four PCOS phenotypes in terms of symptom severity and metabolic comorbidities. Several other studies at the time of publication of our results

have also found similar conclusions regarding phenotype differences (4,144), however ours was the first to include women with only one Rotterdam criterion (1RC) as well as controls.

Regarding the risk of developing IR, we could show that elevated free testosterone levels in serum are the most indicative parameter for its occurrence, even after adjustment for age and BMI. While a previous study by Antonio et al. also found similar results, they did not differentiate between PCOS phenotypes, nor could they show statistically significant ORs after adjustment for BMI and age (8).

In the 1RC group, we could also show fTesto as an important covariate of IR development, though the result was only significant before adjustment for age and BMI, most likely due to the lower sample size (n=75), as the parameter still trended towards significance after adjustment. And while androgen excess has been assessed previously with regard to developing type 2 diabetes or metabolic syndrome in women with and without PCOS (145,146), they did not evaluate IR development as a precursor to the above mentioned metabolic conditions.

Interestingly, compared to other studies (146–148), ours had a lower prevalence of metabolic syndrome or hyperglycemia, despite a high number of women with IR. The average age of our patients, which was 26-28 years in all phenotype groups and the 1RC, with only the control group averaging at 36.7 years, could explain this difference.

More recently, a large cross-sectional study was published assessing PCOS comorbidities and phenotype distribution in almost 9000 women in India (149). While most women included in analysis were healthy controls, 325 women with PCOS and 492 women with one diagnostic criterion, defined as “Pre-PCOS”, were identified, while additional diagnoses in the study were identified via questionnaires. In contrast to our cohort study established from the endocrinology outpatient clinic at the Medical University of Graz, where women were referred to as suspected PCOS cases, their cross-sectional study was more representative of the general population, offering a more in-depth analysis of PCOS prevalence, their phenotypical distribution, as well as the assessment of relevant comorbidities (149).

Using the same phenotype definitions and designations as our cohort study (A-D), 20.2 percent of PCOS women could be designated within phenotype A, expressing all three Rotterdam criteria, 14.3 percent showed HA and OM, but not PCOM, while 40.8 percent had HA and PCOM, but not OM, defined as 8 menstrual cycles or less per year, or a cycle length of 35 days or more. Phenotype D was the second most common at a prevalence of 24.6 percent (149). In contrast, our cohort study had the least PCOS women with phenotype D, which could be explained by their lesser symptom severity compared to other phenotypes, leading to fewer referrals to our outpatient

clinic for further evaluation, as individuals with milder symptoms are less inclined to seek medical attention.

Ganie et al. further reported a prevalence of obesity of 10.9 percent when using WHO definitions, while 43.2 percent of women were considered obese when applying Asian cutoffs (BMI > 27.5 kg/m²). Furthermore, MetS was present in 24.9 percent of women with PCOS and 15.9 percent of women with 1RC, a much higher prevalence of both obesity and MetS compared to our cohort study, possibly due to diverging definitions. Unfortunately, the authors did not differentiate comorbidity prevalence among the different PCOS phenotypes, nor among women with only 1RC (149). This further step could have confirmed our own findings regarding the metabolic risk of hyperandrogenemia, especially free testosterone levels.

4.3 Limitations

Notwithstanding the successful completion of all goals in preparation for the large probiotic RCT, as well as elucidating on the clinical importance of PCOS phenotypes, both the pilot trial and the cohort study have several limitations to consider, both inherent to the topic of research itself (see below) and to the studies themselves.

4.3.1 Limitations of probiotic studies in general

The meta-analyses cited in chapter 1.4.3 highlight many limitations inherent to probiotic research, notwithstanding them showing very promising results in general.

While there are a number of RCTs for any single research topic mentioned above, most meta-analyses are conducted with no more than 1000 participants in total, sometimes across several countries or even continents. Considering the prevalence of the disorders/diseases mentioned above and the high inherent variability of microbiome composition in the general population, more studies with higher sample sizes are required to further validate the data.

Further exacerbating the issue, meta-analyses, while providing a broad overview over the general evidence available, sometimes include studies showing no significant benefit of probiotic use. This discrepancy could be explained by varying methodologies, in particular endpoint and study population selection, the choice and amount of monotherapy or combination of different probiotic strains, as well as any additional prebiotic additives, which may exert metabolic effects

themselves. And these comparisons do not even take into account studies which were never published due to publication bias resulting from negative outcomes.
However, while these factors may explain inter-study differences in results or outcomes, they are themselves again limiting factors for integrating probiotic therapies into evidence-based guidelines. In a meta-analysis containing six to ten different RCTs, every single study protocol will contain vast differences in methodology, limiting their reproducibility and the usefulness of the meta-analysis for clinical practice.
This limitation could be partly based on lacking information regarding microbiome composition before and after intervention in RCTs assessing clinical or biochemical outcomes in human subjects.
Microbiome research, while becoming more cost-efficient over the last two decades, remains resource- and finance-consuming, especially in clinical settings, where high levels of evidence via RCTs and meta-analyses remain paramount. The more people are included, the pricier the sequencing of microbiota and subsequent metagenomics/metabolomics analyses become. Furthermore, including microbiome composition analysis must not be at the detriment of clinical outcome data, as any treatment needs to show a clinical benefit for the patients. In addition, including microbiome sequencing into a study presents an additional risk for the presentability of the data, as probiotic strains included in commercial products may not always be detected with current sequencing methods (150,151).

4.3.2 Statistical significance

An important topic to address regarding the pilot trial is the lack of statistically significant result differences between the three probiotic intervention groups. Importantly, statistical significance in the differences between the intervention groups was not expected or required prior to trial onset.
First, the power of the results was never expected to show statistical significance, as defined by a p lower than 0.05, due to the low sample size of each intervention group (as this was a pilot trial).
Second, all three probiotic interventions had the potential to be similarly effective/ineffective when compared to each other, especially considering their similar composition. This was also the reason why a placebo arm was missing in the pilot trial, since the main goal of this smaller study was never to test the null hypothesis whether the probiotics would have any statistically significant effect on PCOS symptoms.

4.3.3 Open-label bias

The open-label nature of the trial deserves special notice as a potential source of bias, as it opens up the possibility of favoring one probiotic over another and setting it up as the probiotic of choice for the RCT. Luckily, there are several mitigating factors that make this potential bias far less likely. For one, as previously stated, the probiotics all had similar a priori probabilities of improving PCOS parameters and symptoms, and the involved study personnel had no vested interests in promoting one probiotic over the other. All products came from the same manufacturer (Winclove B.V., Netherlands) and distributor (Institut Allergosan), and while there may have been slight differences in manufacturing costs and selling prices between the products, the contract between the various involved parties did not state any payment differences depending on the probiotic used, nor where the industry partners involved in patient recruitment, data collection, analysis or interpretation. They were updated regularly by the principal investigator of the trial Prof. Barbara Obermayer-Pietsch on the current status of the trial and any relevant results and interpretations from the investigative team, which did not include any industry partner employees.

In addition, manipulating a pilot trial involving women with PCOS and three different probiotic interventions and especially predicting which probiotic product might improve PCOS the most would be more difficult than it might initially sound. PCOS is an inherently heterogeneous disorder, as are microbiome changes due to the disorder. High quality evidence on whether probiotics improve PCOS symptoms at all is still missing (which is one of the reasons why the RCT was deemed necessary and relevant). In order to manipulate the pilot trial and promote one probiotic over the others, the manipulating party would have to be aware of all relevant symptoms and biomarkers in a potential participant before they attend the screening visit, and would furthermore have to decide on which probiotic would have the highest likelihood of improving those symptoms, despite lacking any prior evidence of improvement in women with PCOS.

In the case of the pilot study, blinding it for the investigators and participants would have created a much higher financial and logistical burden, as these products including the packaging would have had to be custom made for the pilot trial instead of using the commercial products already in circulation.

For all of the reasons above, the pilot trial was kept open-label.

4.3.4 Lack of probiotic strains in stool sample analyses

Another limitation of the pilot trial was the microbiome composition of the three intervention groups. Importantly, none of the probiotic strains used exclusively in only one of the three products were found as significant OTUs for their respective group. There are several potential reasons for this discrepancy. First, the sample size of the pilot trial was extremely low, making it very difficult to differentiate the probiotic intervention, as interpersonal differences within the same group can be considered as large as those between two separate groups. Second and connected to the first issue, lactic acid bacteria are very common and abundant in most individuals, making a distinction of specific lactic acid producing OTUs very difficult, as most if not all bacterial strains used in the probiotic are already occurring in most individuals. Third, many bacterial strains added to the gut via probiotics do not colonize in the gut, but instead remain in the lumen, thereby limiting the metabolic benefits the individual can receive (152).

To address these issues as well as increase the potential beneficial effects in PCOS, the number of living organisms per portion would be doubled and the list of ingredients would be adapted slightly by Institut Allergosan. In addition, the RCT would incorporate recto-sigmoidoscopies and gastroscopies in order to obtain biopsy samples of the intestinal wall and to test these for microbial strains. This analysis might help differentiate between luminal and gut wall colonization of the probiotic strains used in the intervention.

Further biases might be found in the participants´ diets. As already stated previously, many food products naturally contain probiotic strains including lactic acid producing bacteria, and while dietary intervention has been shown to successfully improve some symptoms of PCOS (see chapter “1.3.1 Lifestyle intervention”), implementing a dietary regime would have complicated the trial setup even more, in addition to further reducing the potential pool of interested women with PCOS via food intolerances or non-adherence to the prescribed dietary regimen. We specifically wanted to test the effectiveness of probiotics independently of the participants´ diets, therefore we did not impose dietary restrictions, nor did we assess dietary intake of macro-/micronutrients and/or probiotic-containing products as study endpoints. Our goal is to prove a clinically relevant effect even in suboptimal or detrimental diets/lifestyles.

4.3.5 Procedural challenges

In most participants, all the materials necessary for study endpoint analysis were able to be collected, including the urine and stool sample collecting at home. Apart from the three dropouts,

only one participant failed to return stool samples after the intervention period due to forgetting their samples at home and living far away from the hospital.

The biggest challenge proved to be measuring insulin levels during the oral glucose tolerance tests (oGTT) at the clinic. In ten participants during the screening visit, the serum blood samples collected for insulin measurements at one or more time points during the oGTT proved to be hemolytic, invalidating the results. Therefore, Insulin AUC was not calculable in these cases. During the second visit, Insulin AUC was incalculable in seven participants, making it the most unreliable study endpoint to calculate. On possible explanation for this phenomenon was the use of venous catheter systems with a too narrow lumen, causing hemolysis if used repeatedly during a 2-hour-period.

In order to address this issue, serum samples for insulin measurements would have to be more carefully collected during the RCT. While the use of a venous catheter during the oral glucose tolerance test was more preferable than single-use blood draws in order to minimize the risk of infections or local skin irritations, wider catheters could be used to reduce the risk of hemolysis in the future.

Another issue that arose was the return of the collected urine and stool samples. After collection, participants had to store the samples at either -18°C (in a household freezer using multiple layers of packaging for hygienic purposes) or at +4°C (in a household fridge, again using additional packaging) in the case of two of the urine samples collected. While most participants lived in the greater Graz area, ensuring that the samples were exposed to warmer temperatures for a maximum of 30 minutes, some participants lived in the suburbs or even further away, risking a thawing of the samples and potentially ruining some of the samples. Luckily, all the collected samples at the pilot trial arrived in a frozen/appropriately cooled condition, notwithstanding the potential for future sample loss due to this issue.

This problem was addressed for the RCT by providing RCT participants cooling packs and an insulated bag in cases were the sample transport from their homes to the outpatient clinic took a longer time.

4.3.6 Cohort study limitations

While we could demonstrate the importance of differentiating between the PCOS phenotypes as well as the impact of hyperandrogenemia and elevated free testosterone in particular on developing IR, the cohort study still has several limitations.

Firstly, the distribution of participants between the phenotypes was very uneven, with phenotypes A and B having many more entries than phenotypes C and D as well as group 1RC. The control group, with which every other group needs to be compared, has the least number of entries, both due to the nature of recruitment (patients are recruited from our endocrinology outpatient clinic, and women only seldom get referred to it without any clinical presentation of PCOS) and the strict definition of PCOS criteria (e.g. modified Ferriman Gallwey scores of 0 to 4 points counting as "normal". While this decision was based on previous literature (118), this definition automatically shifts many women into phenotypes A, B or C or 1RC). Many of our results did not show statistical significance, and it is possible that the different sample sizes of the groups had a significant impact on those results.

Due to the above mentioned preselection bias in our outpatient clinic, the age difference between the control group and the other five groups also presents a challenge. It is difficult to recruit healthy young women in an outpatient clinic designed to treat patient with various symptoms and illnesses. Since the results of this study were published, efforts were taken to recruit more women without any PCOS symptoms as well as women with only one Rotterdam criterion.

The third main limitation regards the measurement of total testosterone via immunoassay, as opposed to liquid chromatography tandem mass spectrometry (LC/MS). LC/MS is unfortunately not readily available at our clinical laboratory, and due to logistical constraints, we could not ship blood samples of every participant to another location for LC/MS measurements. However, in order to mitigate this problem, we had the Endocrine Laboratory at the VU University Medical Center, Amsterdam, Netherlands measure total testosterone in a subset of 113 patients using the LC/MS method and compared their results.

4.4 Impact on future treatment regimens

This PCOS phenotype comparison study underlines the importance of evaluating the not only the presence or absence of PCOS when deciding if a women with PCOS-typical symptoms seeks medical help, but more specifically the PCOS phenotype present (if two or more Rotterdam criteria are met), and in particular if hyperandrogenemia is present even in the absence of other PCOS criteria. Both factors impact the metabolic risk of the patient as well as the potential treatment options. Where possible, oral glucose tolerance tests including insulin measurements should be performed to diagnose IR or type 2 diabetes, and in those cases as well as in cases involving

increased BMI, lifestyle intervention should be recommended and use of metformin or even GLP-1RA should be evaluated.

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