Thesis

MiRNAs in PCOS phenotypes

submitted by

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Graz, 22nd April 2024

Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations 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 "Good Scientific Practice".

Graz, 22nd April 2024 Jonas Höller eh.

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Graz, at 22nd April 2024 Jonas Höller

Zusammenfassung

Einleitung: Das polyzystische Ovarsyndrom (PCOS) eine häufige endokrine Variante, die den Stoffwechsel, die Fortpflanzung und die psychische Gesundheit von Frauen stark beeinträchtigen kann. Die weltweite Prävalenz liegt, je nach den verwendeten Diagnosekriterien, zwischen 4 und 21 %. PCOS ist durch eine Kombination von drei Symptomen gekennzeichnet: klinischer- oder biochemischer Hyperandrogenismus (HA), Oligo- oder Anovulation (AO) und polyzystische Ovarialmorphologie (PCOM). PCOS hat ein breites Spektrum an möglichen Phänotypen, Symptomen und Spätfolgen. Frühere Studien zeigten, dass Frauen, die von PCOS betroffen sind, spezifische Mikro-RNAs (miRNAs) in verschiedenen Untersuchungsmaterialien exprimieren. Ziel dieser Studie war es, veränderte miRNAs, die mit PCOS assoziiert sind, im Serum von Frauen mit spezifischen PCOS-Phänotypen zu untersuchen.

Methoden: Wir führten eine Pilotstudie durch (n = 51), mit 11 Proben für die Phänotypen A, C und D, 10 Proben für Phänotyp B und 8 Proben für die Kontrollgruppe. Im Labor wurden die miRNA-Isolierung, komplementäre DNA (cDNA)-Synthese und Real-time PCR (qPCR) durchgeführt. Als exogene Kontrollen wurden Uni Sp 2/4/5/6 verwendet und mit dem Mittelwert von miR-484 und snu6 als endogenen Kontrollen normalisiert. Nach Prüfung auf Normalverteilung und Varianzhomogenität wurden ein One-way-ANOVA oder ein nichtparametrischer Kruskal-Wallis-Test durchgeführt, gefolgt von Dunnet- und Tukey-Post-hoc-Tests. Darüber hinaus wurde eine Receiver Operating Characteristic (ROC)-Analyse durchgeführt.

Ergebnisse: Frauen mit PCOS wiesen signifikant höhere Hormonspiegel, stärkeren Hirsutismus und unterschiedliche Hormonprofile zwischen den Phänotypen auf. MiR-23a-3p und miR-424- 5p zeigten eine veränderte Expression bei den verschiedenen PCOS-Phänotypen, was auf ein Potenzial zur Unterscheidung zwischen den Phänotypen hinweist. MiR-93-5p zeigte eine geringere Expression bei PCOS im Vergleich zur Kontrollgruppe, was auf einen potenziellen Marker zur Feststellung des PCOS-Status hindeutet.

Diskussion: Das Verständnis der unterschiedlichen Hormon- und miRNA-Profile bei den verschiedenen PCOS-Phänotypen könnte den Weg für personalisierte Behandlungen ebnen, die auf die spezifischen Prozesse zugeschnitten sind, welche mit jedem

Phänotyp verbunden sind. Die identifizierten miRNAs miR-23a-3p und miR-424-5p sind potenzielle Biomarker für die Unterscheidung zwischen spezifischen PCOS-Phänotypen. Diese Ergebnisse tragen zu einem tieferen Verständnis der Heterogenität und Pathophysiologie des PCOS bei und können sich dadurch potenziell auf die Forschung der Diagnose- und Behandlungsstrategien für von PCOS betroffenen Frauen auswirken. Weitere Untersuchungen in größeren Kohorten sind erforderlich, um unsere Ergebnisse und ihre klinische Bedeutung für betroffene Frauen zu klären.

Abstract

Introduction: Polycystic ovary syndrome (PCOS) is a common endocrine condition affecting women's metabolic, reproductive, and psychological health. Global prevalence ranges from 4 to 21%, depending on the diagnostic criteria used. It is characterised by a combination of three key symptoms: clinical or biochemical hyperandrogenism (HA), oligo- or anovulation (AO) and polycystic ovary morphology (PCOM). PCOS has a wide spectrum of different possible phenotypes, symptoms, and complications. Prior studies suggest that women affected by PCOS express specific micro RNAs (miRNAs) in various tissues. The aim of this study was to characterise altered miRNAs associated with PCOS in serum of women with specific PCOS phenotypes.

Methods: We conducted a pilot study (n = 51), with 11 samples for phenotypes A, C and D, 10 samples for phenotype B and 8 samples for the control group. The laboratory methods performed were miRNA isolation, complementary DNA (cDNA) synthesis and quantitative real-time PCR (qPCR) with kits from Qiagen. We used Uni Sp 2/4/5/6 as exogenous controls, and we normalized with the mean of miR-484 and snu6 as endogenous control. After verifying the normal distribution and homogeneity of variances, one-way-ANOVA or a non-parametric Kruskal-Wallis test was performed, followed by Dunnet and Tukey post-hoc tests. The potential diagnostic value for discriminating a specific PCOS phenotype was calculated using area under the curve (AUC) and a receiver-operating characteristic (ROC) analysis. Using TargetScanHuman 8.0 we identified potential target genes of differentially expressed miRNAs.

Results: Women affected by PCOS exhibited significantly higher hormone levels, increased hirsutism scores and distinct hormone profiles across phenotypes. MiR-23a-3p and miR-424-5p showed altered expression in PCOS phenotypes, indicating a potential for differentiating between phenotypes. MiR-93-5p displayed lower expression in PCOS compared with control group, suggesting a potential marker to discriminate PCOS status.

Discussion: Understanding the differential hormone and miRNA profiles across various PCOS phenotypes could pave the way for personalized treatments for specific imbalances or pathways associated with each phenotype. The identified miRNAs, such

as miR-23a-3p and miR-424-5p, hold promise as potential biomarkers for distinguishing between specific PCOS phenotypes. These findings can contribute to a deeper understanding of the heterogeneity of PCOS, influencing future research and potentially improving diagnostic and treatment strategies for women affected by PCOS. More research in larger cohorts is important to reevaluate our findings and its clinical implication for affected women.

Disclosures

This thesis served as a basis for publications of original data in the International Journal of Molecular Sciences [1]. I myself acted as co-author. I have not included any results or figures in this thesis that originate from other co-authors without my contribution.

Since I took part in the writing of the manuscript, I have included some parts in the present thesis and cited them accordingly. Corresponding passages are cited and information about the publication is provided in the references.

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Contents

List of Abbreviations

Symbols

25OHD 25-hydroxy-vitamin D. 27

A

ACTH adrenocorticotropic hormone. 6, 27
AGE advanced glycation end-product. 7
AGO argonaute protein. 17
ALT alanine aminotransferase. 27
AMH anti-müllerian hormone. 5, 6, 9, 11, 12, 15, 20, 22, 41, 42, 56
AO oligo- or anovulation. D, 1, 3, 6, 11, 12, 13, 14, 26
AST aspartate aminotransferase. 27
AUC area under the curve. D, 38, 39, 46, 47, 48, 49, 57

B

BMI body mass index. 7, 8, 14, 16, 20, 22, 27, 38, 41, 42, 58
BPA Bisphenol A. 5

C

cDNA complementary DNA. D, 31, 33, 34, 35
CI confidence interval. 46, 47, 48, 49
COCP combined oral contraceptive pill. 14

D

DHEA dehydroepiandrosterone. 4
DHEAS dehydroepiandrosterone sulfate. 26, 27, 41, 42, 56
DMT2 diabetes mellitus type 2. 2, 3, 7, 10, 15

E

EDCs endocrine disruptors. 5

F

FSH follicle-stimulating hormone. 6, 7, 8, 9, 13, 15, 27, 41, 56

fT3 free triiodothyronine. 27
fT4 free thyroxine. 27

G

GABA γ-aminobutyric acid. 6
GDM gestational diabetes mellitus. 3, 10
GGT γ-glutamyl transferase. 27
GLP-1 glucagon-like peptide-1. 8
GnRH gonadotropin-releasing hormone. 5, 6, 9

H

HA hyperandrogenism. D, 1, 2, 5, 6, 7, 8, 11, 12, 14, 15, 26, 27, 57
HbA1c glycated hemoglobin. 27
HDL high-density lipoprotein. 3, 18, 27
HGH human growth hormone. 27
HHAA hypothalamus-hypophysis-adrenal axis. 6, 7
HHOA hypothalamus-hypophysis-ovary axis. 7
HOMA homeostatic model assessment. 12
hsCRP high-sensitivity C-reactive protein. 27

I

IGF-1 insulin-like growth factor 1. 27
IGT impaired glucose tolerance. 2
IL-6 interleukin 6. 8
IL-22 interleukin 22. 8
IR insulin resistance. 2, 3, 4, 5, 6, 7, 8, 10, 12, 15, 20, 22, 50, 51, 52, 54, 57

L

LDL low-density lipoprotein. 3, 18, 27
LH luteinizing hormone. 6, 7, 8, 9, 12, 13, 15, 27, 41, 56
LHCGR luteinizing hormone/choriogonadotropin receptor. 22

M

mFGS modified Ferriman-Gallwey score [3]. vii, 2, 26, 41, 42, 57

miRNA micro RNA. D, vii, viii, 16, 17, 18, 19, 20, 21, 22, 23, 24, 28, 29, 30, 31, 32, 34, 38, 39, 40, 43, 44, 45, 46, 47, 48, 49, 50, 52, 53, 55, 56, 57, 58, 59, 60
mRNA messenger RNA. 16, 17, 18, 21

O

OCD obsessive-compulsive disorder. 4, 10

P

PCOM polycystic ovary morphology. D, 1, 9, 11, 12, 13, 26, 42
PCOS polycystic ovary syndrome. C, D, E, vii, viii, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 19, 20, 22, 23, 24, 25, 26, 27, 37, 41, 42, 45, 46, 47, 48, 49, 50, 52, 53, 55, 56, 57, 58, 59, 60
PCR polymerase chain reaction. 34, 38
PTH parathyroid hormone. 27

R

Ran-GTP ras- related nuclear protein-guanosine triphosphate. 17
RISC rNA-induced silencing complex. 17, 18, 21
ROC receiver-operating characteristic. D, 38, 39, 46, 47, 49
ROS reactive oxygen species. 8

S

SCFA short chain fatty acid. 8
SHBG sex hormone-binding globulin. 6, 13, 20, 22, 27
shRNA short hairpin RNA. 17, 21

T

T testosterone. 5, 6, 8, 12, 20, 22, 26, 27, 41, 42, 56
TMAO trimethylamine N-oxide. 13
TNF tumor necrosis factor. 7, 8
TRBP transactivator RNA binding protein. 17, 21
TSH thyroid-stimulating hormone. 27

List of Figures

List of Tables

Chapter 1

Introduction

1.1 Theoretical background

1.1.1 Polycystic Ovary Syndrome

Polycystic ovary syndrome (PCOS) is a common endocrine condition affecting women's metabolic, reproductive, and psychological health. Global prevalence ranges from 4 to 21%, depending on the diagnostic criteria used [4]. It is characterized by a combination of key symptoms: clinical or biochemical hyperandrogenism (HA), oligo- or anovulation (AO) and polycystic ovary morphology (PCOM). These three criteria form the so-called Rotterdam criteria, whereby at least two of the three criteria must be met, and other causes have to be reliably excluded to establish a diagnosis of PCOS [5]. Four phenotypes result from the Rotterdam criteria: A, B, C and D. See table 1.1. These are also the phenotypes used in this study.

Phenotype	HA	AO	PCOM
A	✓	✓	✓
B	✓	✓	×
C	✓	×	✓
D	×	✓	✓

Table 1.1: The PCOS phenotypes resulting from the Rotterdam criteria. Hyperandrogenism (HA); Oligo- or anovulation (AO); Polycystic ovary morphology (PCOM)

1.1.1.1 Symptoms

PCOS is a chronic and extremely heterogeneous condition that can significantly impact the lives of affected women. Thereby, the possible clinical manifestations can be divided into 3 groups: metabolic features, reproductive manifestations, and psychological

consequences. Metabolically, PCOS can manifest with obesity, insulin resistance (IR), increased risk of diabetes mellitus type 2 (DMT2) and/or dyslipidemia, among other symptoms further explained below. Reproductively, PCOS is the most common cause of an-ovulatory infertility and may cause dysfunctional uterine bleeding in addition to anovulation [6]. Psychological implications or symptoms of PCOS may include low self-esteem, negative body image, depression, or anxiety disorders [6–8].

Categorising hirsutism into these three groups proves difficult, though mentioning it separately remains essential, considering it is the most recognised symptom of PCOS [9]. Hirsutism can be defined as an abnormal amount of terminal hairs with male pattern in women and is the main expression of HA. Also, acne, oily skin, virilization, and male pattern alopecia are clinical symptoms of HA and PCOS [3, 6, 10, 11]. Hirsutism can be assessed with modified Ferriman-Gallwey score [3] (mFGS), see also figure 1.1. PCOS women of phenotypes A, B and C exhibit HA.

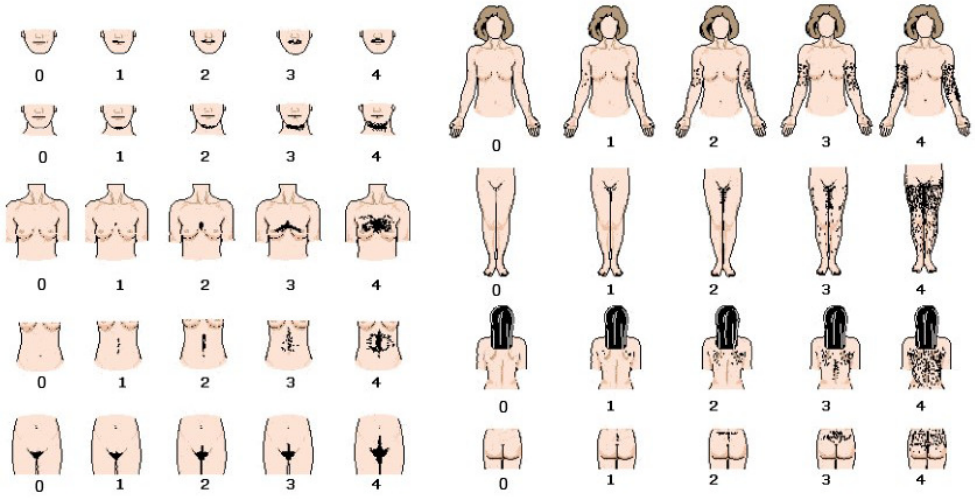


Figure 1.1: The Modified Ferriman-Gallwey score [3] concerns nine body regions, which are scored from zero (no terminal hair) to four (abundant terminal hair), the overall score is calculated through the addition of each area [12].

- **Metabolic**: There is evidence confirming an increased risk of impaired glucose tolerance (IGT) and DMT2 in both pre- and post-menopausal women with PCOS, even suggesting that they are a high-risk group. Also, young women with PCOS are already at risk for metabolic syndrome and dyslipidemia [13]. There are diverse factors contributing to the metabolic risks in PCOS.

Obesity, overweight, and difficulty losing weight are very common and are cited by patients as the most concerning features of PCOS. Another cardiovascular risk factor is associated with PCOS, namely high blood pressure [13].

Insulin resistance can be defined as a reduced responsiveness of insulin-targeting tissues to normal levels of insulin or, in other words, as impaired insulin sensitivity [14] and is often connected with compensatory hyperinsulinemia and later hyperglycaemia [15]. High insulin levels directly and indirectly affect vascular calcification, contributing enormously to the cardiovascular risk [16]. IR is associated with PCOS, most notably with hyperandrogenic phenotypes, but not only (the prevalence is about 50% to 80%) [6]. IR is an important risk factor for the development of metabolic syndrome, DMT2, cardiovascular disease and cancer [15].

In addition, high low-density lipoprotein (LDL) and low high-density lipoprotein (HDL) are present in women with PCOS (in combination with obesity) and are likley to persist beyond menopause [13].

Thus, PCOS is associated with a higher risk for gestational diabetes mellitus (GDM), pregnancy-induced hypertension and pre-eclampsia, which are known to lead to an increased long-term cardio-metabolic risk. Relatives of women affected by PCOS also had a higher prevalence of metabolic syndrome and dislipidaemia [13].

- **Reproductive**: PCOS women (all phenotypes except C) have ovarian dysfunction which manifests with dys- or amenorrhea, resulting from chronic anovulation. It is important to note that dysfunctional uterine bleeding can mimic regular menstrual cycles, and that oral contraceptives can mask irregular cycles. AO can lead to infertility, which is also a significant symptom of PCOS, considering that many PCOS women are diagnosed in fertility clinics [17]. Prevalence of infertility in women with PCOS ranges from 70% to 80% [18, 19]. Then again, a new genetic study found that considering the family size achieved, PCOS appears to be a state of delayed fertility rather than infertility [20].

- **Psychological**: Apart from the fact that PCOS can have an impact on the quality of life, it can also affect psychological health. Due to the characteristics of PCOS (acne, hirsutism, obesity) women are more likely to develop low self-esteem, negative body image, anxiety, and depression [6], as well as bipolar

disorder and obsessive-compulsive disorder (OCD). In addition, symptoms of depression, anxiety, and somatization appear to be more severe in women affected by PCOS. It appears that distress related to PCOS symptoms may determine mood issues. Depressive symptoms in women with PCOS have been shown to correlate with androgen levels [8] and hirsutism [21]. The median prevalence of depressive symptoms among women affected with PCOS is 36.6% and 41.9% for anxiety symptoms [13]. Moreover, depression and anxiety may coexist with other mood disorders. This potential coexistence may further compromise the health and well-being of these patients [8].

1.1.1.2 Aetiology

Both genetic and environmental factors are assumed to play a role in the development of PCOS, although no single gene has yet been identified to specifically cause PCOS or to have a relevant influence on its development [7, 22, 23]. This also explains why there are different phenotypes. It seems paradoxical, but it is suggested that PCOS may have been an evolutionary advantage during earlier times. Smaller family size as a result of fewer births due to anovulation or the increased muscle mass, bone mineral density, the protective role of adrenal dehydroepiandrosterone (DHEA) on the immune system and the elevated energy storage capacities due to IR may have had a positive effect on the chances of survival in the rough conditions of the hunter-gatherer lifestyle [24]. A 2006 Dutch family study quantified the genetic influence on the pathogenesis of PCOS, finding that hereditary factors were strongly influential [25]. Subsequent studies demonstrated an association with PCOS for diverse gene loci. However, varying results were found in different populations [26–33]. A new 2024 study involving approximately 500,000 participants from the International PCOS Genetics Consortium has identified 29 independent loci and 31 proteins associated with PCOS. Besides, a polygenetic risk score was calculated and associated with metabolic outcomes. These genomic loci not only relate to PCOS but also with age at menopause, suggesting a link between reproductive longevity, ovarian reserve, and DNA repair efficiency [20].

One environmental factor discussed in relation to the development of PCOS is the intrauterine influence of androgens on the unborn child. The programming of genes involved in ovarian steroid synthesis, insulin metabolism, gonadotropin secretion, and ovarian follicular maturation influences the potential development of PCOS in adulthood [7, 34, 35]. Two animal models showed increasing evidence that excess

testosterone (T) in human fetal development could promote PCOS in adulthood [36]. Also, prenatal anti-müllerian hormone (AMH) exposure hyperactivates gonadotropin-releasing hormone (GnRH) neurons [37], which then potentially initiate the pathological cycle (see: 1.1.1.3). This adverse intrauterine environment does not only affect the fetus itself but also the fetal germ cells, which may also undergo epigenetic changes [38]. The intrauterine environment has also been associated with the development of various PCOS phenotypes [22].

Similarly, medication intake has been discussed in relation to the development of PCOS. The antiepileptic drug valproic acid may be associated with PCOS [22]. According to a 2011 meta-analysis, female patients with epilepsy who were treated with valproic acid had a higher prevalence of PCOS than women who were administered alternative antiepileptic medications [39]. A 2018 case study suggests a causal and reversible correlation [40].

A further environmental determinant that could be relevant concerns toxins [22]. The endocrine disruptors (EDCs) seem to be particularly significant. Thereby, it is hypothesised that EDCs increase or aggravate the likelihood of developing PCOS [7, 41]. Bisphenol A (BPA), as a representative of this group of chemicals, is suspected to be involved in the pathogenesis of HA and hyperinsulinemia. Furthermore, maternal exposure to BPA during pregnancy could lead to PCOS in the female offspring [42]. In addition, there is speculation about infectious causes [22]. In this context, a Turkish study found an association between adenovirus infection and obesity in PCOS [43].

New research suggests that genetic non-alcoholic fatty liver is associated with a higher risk of developing PCOS [44]. Besides, recent evidence also points to a causal association between obesity, central fat, IR and PCOS [45]. Underlining the relevance of obesity and diet: weight exacerbates many/almost all PCOS symptoms. There is an improvement of PCOS symptoms with a minimal weight loss of 5%, with simultaneous improvements in circulating androgens, gonadotropins, and IR. Intervention programmes with diet and exercise achieved excellent results. Food quality and type of diet may also alter the PCOS phenotype, possibly interacting with different genetic patterns [22]. Obesity is a powerful amplifying factor for many aspects of PCOS, which are not limited to its relationship with the development of IR and hyperinsulinemia [46]. On a genetic background, also, high-sugar foods in the Western diet may be inducers of PCOS through multiple interrelated mechanisms: creating imbalance of the

gut microbiome and also triggering chronic inflammation, IR as well as the production of androgens [47] (see also 1.1.1.3).

1.1.1.3 Pathogenesis

The pathogenesis of PCOS is a complex relation of several factors. Recent literature proposes the following pathophysiological processes of PCOS, when reproductive and metabolic phenotype are considered. Increased frequency of GnRH release leads to increased luteinizing hormone (LH) secretion [38, 46, 48, 49]. γ-aminobutyric acid (GABA) is considered an inhibitory neurotransmitter in the brain, but it might exert a stimulatory effect on GnRH neurons. In a mouse model, activation of GABA neurons induced a PCOS-like phenotype, indicating that GABA may be a (co)stimulatory factor of the increased GnRH pulse in PCOS [50]. Thus, the GnRH pulse is resistant to negative feedback from ovarian steroids (probably mediated by androgen excess). LH then stimulates the production of T in theca cells [38, 46]. Furthermore, over IGF -1 LH stimulates the LH receptor binding [50].

Hyperinsulinemia (resulting from IR independent of obesity) stimulates theca cell T production, exacerbates LH hyper-secretion [38] and decreases the production of sex hormone-binding globulin (SHBG) [51], which further increases HA by elevating the bioavailability of T, upregulates the LH receptor in theca cells, inhibiting follicular maturation and growth leading to AO [38]. Additionally, it reinforces adrenal glands' androgen production by potentially activating hypothalamus-hypophysis-adrenal axis (HHAA) and sensitising the adrenal cortex to adrenocorticotropic hormone (ACTH) stimulation [46]. Regarding IR, this effect is explained by the fact that only the glucose metabolism is insulin resistant, whereas the effects of insulin on stereogenesis remain unchanged [52, 53]. Moreover, adipocytes are thought to be resistant to insulin [46]. Furthermore, HA may also affect insulin sensitivity [54], creating a vicious cycle.

T is incompletely aromatized due to a relative follicle-stimulating hormone (FSH) deficiency (lower LH/FSH ratio) [48]. This results in an excessive production of AMH. AMH inhibits CYP19A1, resulting in less aromatase, which leads to obstruction of the conversion of androgens to oestrogens, thereby increasing androgen levels. In addition, AMH enhances GnRH neuron activity, stimulating LH secretion [38], and since lower AMH levels are necessary for folliculogenesis, it plays a crucial role in AO in PCOS women [46]. T is also increased by other enzyme activity in polycystic ovaries [48].

Besides that, obesity, diet and visceral fat have also been implicated in the pathophysiology of PCOS. In this context, hyperlipidemic and low-fibre diets have been associated with HA. This effect is possibly caused by diet-induced hyperinsulinemia with all the previously mentioned effects.

Another possible mechanism is a direct influence of diet on ovarian processes. Advanced glycation end-products (AGEs) deriving from disrupted carbohydrate metabolism, typically present in Western diets, induce oxidative stress and lead to damage to all ovarian cell types, altering their functionality.

Obesity directly affects hormones through the expression of aromatase in adipocytes. More aromatase activity leads to higher oestrogen synthesis, with oestrogens then stimulating LH and inhibiting FSH secretion, further decreasing the LH/FSH ratio. This leads to an amplification of this hyperestrogenemia-hyperandrogenemia cycle in obese PCOS women. Additionally, a large amount of visceral fat can increase the production of androgens in the adrenal gland by leading to high levels of cortisol, which triggers the activation of HHAA. This not only increases the androgens in the adrenal gland, but also the glucocorticoids that induce the expression of aromatase [46].

Another factor is leptin, a hormone secreted primarily by adipose tissue. In addition to its multiple neuroendocrine, reproductive and immune functions, leptin appears to be a permissive factor in the functioning of the hypothalamus-hypophysis-ovary axis (HHOA). High leptin levels indirectly stimulate LH secretion and have a direct negative effect on folliculogenesis. Hyperleptinemia also promotes inflammation through increased production of pro-inflammatory cytokines. Constantly high leptin levels also lead to selective leptin resistance, the diminution of neuroendocrine effects, which promotes obesity through defective appetite suppression [46]. In this context, adiponectin is also a hormone secreted primarily by the adipose tissue. In women affected by PCOS, the levels of this hormone are significantly lower. Low levels of this hormone are associated with obesity, IR, metabolic syndrome and DMT2. In PCOS adiponectin is low independent of body mass index (BMI), it appears to be due to a unique relationship with androgen levels. On the other hand, high levels of adiponectin are a protective factor for this condition. Levels of adiponectin are known to be inversely related to adipocyte mass [55].

It is known that PCOS women have higher serum inflammatory markers. One of these is tumor necrosis factor (TNF). TNF decreases insulin sensitivity and stimulates theca

cell steroidogenesis as well as follicular atresia. The hyperglycaemia resulting from IR contributes to inflammation in PCOS by reactive oxygen species (ROS) generating circulating mononuclear cells that oxidise glucose. This oxidative stress also increases ovarian steroidogenesis [46].

The gut microbiome plays a role in PCOS physiology, in particular women affected by PCOS tend to have reduced diversity and altered phylogenetic parameters [56]. This may be an effect of the known reproductive dysfunction and metabolic dysregulation. Alternatively, aberrant sex hormones in PCOS may impact the gut microbiome. Besides, the gut microbiota may also modulate neurotransmitter levels in the brain. In women with PCOS short chain fatty acids (SCFAs) levels are significantly lower. SCFAs are gut metabolites and are linked to the maintenance of the intestinal barrier and immune homeostasis. Bifidobacterium lactis V9 can increase SCFAs levels via promoting SCFAs-producing microbiota, which is associated with a mediation effect on hypothalamus and appears to lead to decreased LH and LH/FSH levels. In an animal model, A. muciniphila increased the secretion of another gut metabolite, the glucagon-like peptide-1 (GLP-1), with subsequent improvement of glucose homeostasis [50]. Another implicated intestinal metabolite is bile acid. It has been reported that women with PCOS also have elevated circulating conjugated primary bile acid levels, which are positively correlated with HA [57]. This alteration may result from an elevated Bacteroides vulgatus population, reported to be present in women affected by PCOS. From an experiment with faecal transplantation in mice emerges, that elevated Bacteroides vulgatus leads to lower bile acids, ovarian dysfunction, IR, reduced Interleukin 22 (IL-22) secretion and infertility [58].

Also, intake of phytoestrogens through soy may play a role in PCOS physiology, since they are structurally similar to endogenous oestrogens and improve the reproductive hormonal profiles [59].

Emerging research seems to demonstrate lower neuropeptide galanin levels in women affected by PCOS, which appears to be correlated with serum TNF-α and interleukin 6 (IL-6) levels, since these were normalized by galanin treatment [50]. T acts in periphery to produce signs of androgen excess. T can be aromatized extragonadally to oestradiol, resulting in estrogenic effects on the endometrium in addition to the effects mentioned above [48]. Researchers are still debating whether hyperinsulinemia or HA are the main dysregulation in PCOS, but newest research points to hyperinsulinemia and BMI as the major causal risk factors [20].

Furthermore, it is still not clear whether the abnormal GnRH pulse is a neuronal starting point of the syndrome or rather a mechanism secondary to the effects of reproductive and metabolic factors in combination with an imbalanced immune system and intestinal microbiome [50].

Pathophysiology goes hand in hand with aetiology. The aetiological factors may arise at different stages of the pathophysiological cycle. In particular, by affecting insulin and/or androgens. Women with PCOS have their custom pathophysiological pattern, which rarely includes all off the above-mentioned factors.

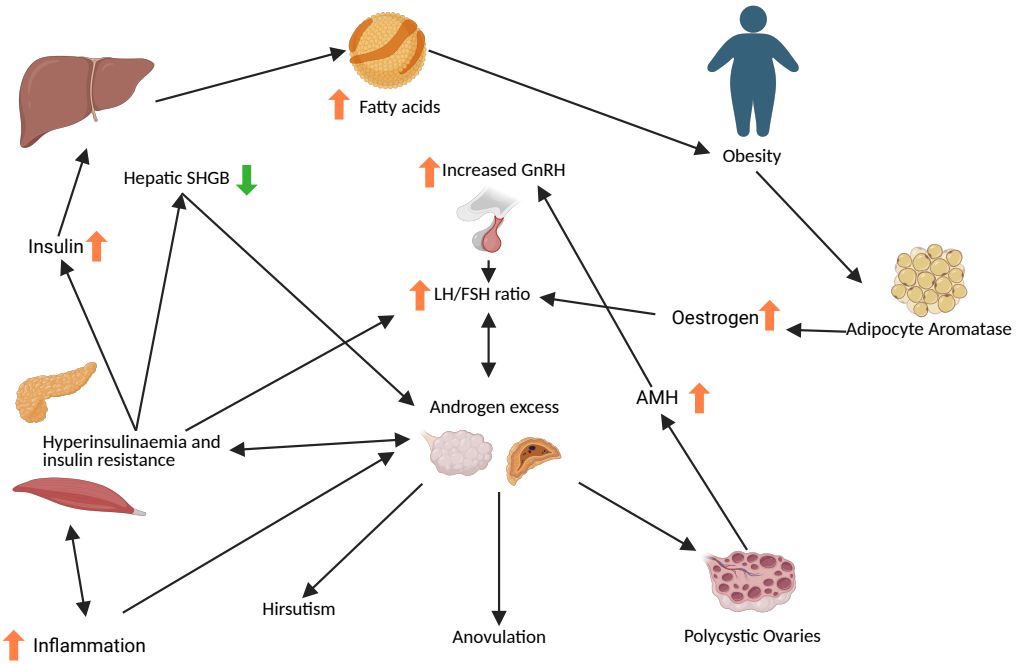


Figure 1.2: Illustration of the complex interplay of hormonal and metabolic factors implicated in PCOS pathophysiology. It is shown how various elements like increased fatty acids, obesity, and insulin resistance can lead to an imbalance in LH/FSH ratio, oestrogen and AMH, leading to conditions like hyperinsulinaemia, hirsutism, anovulation and PCOM.
Created with BioRender.com

1.1.1.4 Complications

PCOS can develop from a reproductive disorder at a young age to a long-term metabolic and cardiovascular disease [17], but without evident increase of related morbidity and mortality [60].

The elevated frequency of IR and hyperinsulinemia in women affected by PCOS act as precursors of GDM and predispose to DMT2 [6], which is an important risk factor for cardiovascular events [61]. Generally, women with PCOS suffer from an increased risk (by 40 %) of GDM, but also the risk for further pregnancy complications is increased [62]. Women with PCOS are more likely to be obese than controls without PCOS, due to a predisposition to accumulate central fat. Obesity aggravates many symptoms of PCOS. Furthermore, mixed hyperlipidemia is very common in women with PCOS. There is also an elevated prevalence of hypertension, endothelial dysfunction and inflammation [6]. The above are all further risk factors for cardiovascular events [63]. In addition to these traditional risk factors, there is evidence of increased subclinical atherosclerosis [13]. As shown in 1.1.1.3, there is a complex relation between these factors. Women with PCOS are also at increased risk for endometrial cancer [64] and other neoplasms such as breast and ovarian cancer [65]. Recurrent anovulation in PCOS leads to uncontrolled oestrogen exposure of the endometrium [6]. Whilst women with PCOS are often infertile, if they remain pregnant, they are at increased risk of developing GDM, pregnancy-related hypertension and pre-eclampsia [6]. Regarding mental disorders, PCOS women have an increased risk of being diagnosed with depression, anxiety, bipolar disorder and OCD. In addition, they have more severe symptoms of depression, anxiety, OCD and somatisation [8]. An association between PCOS and lower urinary tract symptoms has also been observed [66]. In conclusion, the possible long-term health complications of PCOS include: obesity, IR with eventual GDM and/or DMT2, glucose intolerance, hypertension, metabolic syndrome, cardiovascular disease, obstructive sleep apnoea, endometrial cancer [64], breast and ovarian cancer [65], pregnancy complications, hyperlipidaemia acting as an additional factor for cardiovascular complications [6] as well as psychological disorders [64].

1.1.1.5 Diagnosis

In 1935, a case series of women with AO, hirsutism, and bilateral PCOM [67] was published. The first international conference on PCOS was held in Bethesda at the National Institutes of Health (United States of America) in 1990. There, PCOS was defined as chronic AO with clinical and/or biochemical HA [68]. In 2003, the so-called Rotterdam criteria were developed: HA, AO and PCOM, whereby at least two of the three criteria must be met and other causes must be reliably excluded to establish a diagnosis of PCOS [5]. In 2009, the Androgen Excess and PCOS Society stated, based on the available data, that PCOS should be defined by HA(clinical and/or biochemical) and ovarian dysfunction (AO and/or PCOM) after excluding related disorders. Thus, in relation to the Rotterdam criteria, HA is mandatory for the diagnosis [69]. The diagnostic algorithm in the most recent guidelines from 2023 is still based on the Rotterdam criteria [70]:

- **Step 1:** If AO and clinical HA
  - Diagnosis of PCOS
- **Step 2:** If clinical HA is non present
  - Test for biochemical HA and if present PCOS can be diagnosed
- **Step 3:** If only AO or HA
  - Adolescents: ultrasound is not indicated, the patient is considered at risk of PCOS
  - Adults: ultrasound for PCOM or test for elevated AMH levels and if present PCOS can be diagnosed

As previously mentioned, diagnosing PCOS in 2023 is still necessarily a diagnosis per exclusion. It is fundamental to rule out other endocrine pathologies that mimic PCOS [70]. These diseases are: adrenal hyperplasia, Cushing's syndrome, androgen-producing neoplasms, 21-hydroxylase deficiency and drug-induced androgen excess. Moreover, clinicians need to rule out other ovulatory dysfunctions such as thyroid dysfunction and hyperprolactinemia [71].

1.1.1.6 Phenotypes

As already illustrated in table 1.1, the Rotterdam criteria lead to four phenotypes. This classification is relevant for the assessment of the risk of metabolic complications. The four phenotypes differ significantly in terms of levels of LH, AMH, fasting blood sugar, total T concentration [72], AMH and homeostatic model assessment (HOMA)-IR levels [73]. Women with HA (phenotypes A,B and C) are at a higher risk for developing insulin resistance compared to the phenotypes without HA; even women with HA who do not fully meet the diagnostic criteria for PCOS are at higher risk [74, 75].

- **Phenotype A (complete or type I classic PCOS)**: Phenotype A consists of HA, PCOM and AO. This can lead to symptoms such as hirsutism, acne, and male-pattern alopecia, as well as irregular menstrual cycles, which can manifest as infrequent periods or prolonged cycles. This phenotype has a higher risk of metabolic issues, including IR, dyslipidemia, and an increased prevalence of metabolic syndrome when compared to the others [76]. Patients with type I or II of classic PCOS (A and B) show higher values for BMI, waist circumference, Ferriman-Gallwey-Lorenzo scores, levels of testosterone, and insulin [77]. The prevalence of phenotype A is about 50% in clinical populations and about 19% in unselected populations [78].
- **Phenotype B (type II classic PCOS)**: Phenotype B consists of HA and AO. Similar to phenotype A, women have symptoms such as hirsutism, acne and irregular menstrual cycles. Phenotypes A and B are very similar, but differ in the absence of PCOM in B and, for example, in LH and LH/FSH ratios (higher in A than in B) [77]. The prevalence of this phenotype is about 13% in clinical populations and about 25% in unselected populations [78].
- **Phenotype C (Ovulatory PCOS)**: Phenotype C consists of HA and PCOM. Similar to phenotypes A and B, these individuals also have elevated androgen levels, leading to symptoms like hirsutism and acne. PCOM is present although AO is absent. This distinguishes them from classic PCOS. Women with phenotype C have LH and LH/FSH ratios similar to normal controls but lower than patients with phenotype A, B and D [77]. The prevalence of this phenotype is about 14% in clinical populations and about 34% in unselected populations [78].

- **Phenotype D (Normoandrogenic PCOS)**: Phenotype D consists of AO and PCOM. These women have a higher prevalence of normal body mass index, insulin sensitivity, waist circumference and free androgen index[77]. Compared to classic PCOS (A + B), women with this phenotype have a lower LH/FSH ratio with altered levels of thyroid hormones and SHBG. Affected women show normal androgen levels with altered endocrine levels and defective metabolic conditions (though less severe than classic PCOS) [79]. In fact, these women have a metabolic profile similar to women without PCOS. The presence of chronic AO and some endocrine aberrations may suggest an underlying reproductive abnormality [80]. Plasma trimethylamine N-oxide (TMAO) levels are elevated in phenotype D, the relevance of this is still unclear [81]. The prevalence of this phenotype is about 17% in clinical populations and about 19% in unselected populations [78].

A comparison of the prevalences of the clinical and unselected populations [78] shows that phenotype A is diagnosed most frequently, while the other phenotypes are more common in unselected populations than in clinical populations. This indicates that they tend to be underdiagnosed more often.

This classification is important when considering that women with the classic PCOS phenotype (A + B) have a poorer profile of metabolic and CV risk factors compared with women with non-classic PCOS phenotypes (C + D) [80, 82]. These phenotypes can also change. For example, the production of androgens may decrease due to ageing of the ovaries, or the production of the adrenal glands may deteriorate over time. In this example, a phenotype A can convert to a D. Also, hirsutism and acne are less common with increased age [83]. In this context, it is important to underline that self-treatment of hirsutism is common and may improve with increased age, limiting its evaluation [70]. Furthermore, ovarian volume and follicle number can also decrease with age. Similar to the change in phenotype, it is also possible that women no longer meet the diagnostic criteria for PCOS [83]. PCOS phenotype group distribution changes over time. The prevalence of phenotype A decreases and the prevalence of phenotype C increases.

Besides, in many patients, the diagnosis of PCOS can no longer be made. The age effect indicates that menstrual cycles become more regular, serum androgen levels decrease and PCOM improves [84]. Even if these patients no longer fulfil the criteria, it certainly cannot imply that they no longer have PCOS; the diagnosis is to be con-

sidered as lifelong [70]. Other sources even state that it is important to mention that the health risks associated with PCOS are lifelong. Disturbed glucose metabolism, increased androgens and chronic inflammation observed in women affected by PCOS before menopause persist also after menopause [85]. Whether a woman affected by PCOS belongs to phenotype A, C or D has no influence on the morphology of the oocytes [86]. Identifying the phenotype of patients with PCOS-related infertility can help in evaluating the severity of the condition and the fertility outcomes [76].

1.1.1.7 Therapy

Since PCOS is a very heterogeneous condition, with each woman being characterized by a specific combination of symptoms and many pathophysiological mechanisms to be considered, it seems clear that treatment must be individualized and in most cases multifactorial.

In this sense, the 2023 guidelines for diagnosis and treatment of PCOS also indicate that treatment should be tailored to the patient's symptoms and their perception, the minimization of future risks, the impact on quality of live, the key concerns as well as the physician's therapeutic goals, as there is no single specific therapy [70].

Lifestyle intervention should be recommended for all women affected by PCOS, whereby goals and priorities can be individualised in accordance with the patient. Lifestyle management can include behavioural strategies, healthy diet and exercises to improve metabolic and psychological health, but not only consistent lifestyle intervention that also has an impact on the other symptoms of PCOS [70].

For the treatment of non-fertility indications in combination with lifestyle intervention, combined oral contraceptive pill (COCP)is the first-line pharmacological therapy for HA and AO, after screening for contraindications. Whereby additional cosmetic therapy over 6 months is necessary for hirsutism. In the second line, there are three options: the first is to add Metformin to COCP, although this combination offers little additional benefit over Metformin or COCP alone; this is most beneficial in higher metabolic risk groups. The second possibility is to add an anti-androgen such as spironolactone or flutamide to COCP; this should be considered in women with persistent hirsutism after first-line treatment. The third option is the use of Metformin alone, which should be considered in terms of weight, hormonal balance and metabolism, being most useful in higher BMI groups and metabolic high-risk groups. In addition, in adult women

with higher weight, anti-obesity medication may be considered in addition to lifestyle intervention [70].

Also, for fertility indications, the first step is to take lifestyle interventions for optimal health before conception. First-line pharmacological treatment is the aromatase inhibitor letrozole or (though with less evidence) clomiphene citrate in combination with metformin. If ovulation is still not detected, the second-line pharmacological option is the use of gonadotropins under constant ultrasound monitoring. Should ovulation still not be detected, the third line of treatment involves in vitro fertilisation [70].

Psychological therapy is recommended for the treatment of PCOS-related psychological symptoms (see 1.1.1.1), and antidepressants as well as anxiolytics may be considered as second-line treatment based on the guidelines for the general population [70].

Apart from the guidelines, it is also recommended to supplement vitamin D if the patient has a deficiency. Vitamin D can improve IR, lipid metabolism, reduce circulating androgens and lead to a better response to other therapies [87]. A randomized controlled trial from 2021 also suggests the neurokinin-3 receptor antagonist fezolinetant as a therapy, since it had a sustained effect on suppressing HA and lowering the LH/FSH ratio [88]. Probiotic dietary intervention may also be a future therapy; a recent randomised controlled trial was started in 2020 [89]. Furthermore, an European Union project is underway in collaboration with the Medical University of Graz for a phase II clinical trial with pioglitazone, metformin and spironolactone as a triple combination in a single tablet [90, 91].

1.1.1.8 Male phenotype

Indications are growing that there may also in fact exist a male form of PCOS [38]. Male offspring of women affected by PCOS were more susceptible to developing similar metabolic abnormalities later in life [92]. There also appears to be an increased risk of cardiovascular events in fathers of women affected by PCOS [93]. Male first degree relatives of women affected by PCOS have been reported with elevated insulin levels [94], increased prevalence of IR, DMT2 [95], metabolic syndrome and obesity [96]. Also, AMH, LH, and FSH levels were elevated in adult male relatives of affected women [97], making it plausible to speculate that they may have altered testicular function and altered neuroendocrine regulation of gonadotropins [98]. In a large genetic study, a common genetic pathway was found between women affected by PCOS and male

pattern hair loss, indicating a male form of the condition [99]. Furthermore, genetic risk factors associated with PCOS are also known to affect men. This confirms that ovaries are not necessarily required to develop a PCOS- like phenotype [100]. Additionally, a PCOS polygenetic risk score calculation shows an association with higher BMI and increased risk of obesity in both women and men [20].

Although the male form of PCOS can be considered a fact, further research is needed.

In this context, it is also important to consider androgenic effluvium in men, since hormonal similarities have been reported in men with early-onset androgenic alopecia and in male relatives of women with PCOS [101]. Male androgenetic alopecia occurs in up to half of men by the age 50, typically affecting specific areas of the scalp and impacting self-image significantly. The cause is a genetic susceptibility to androgens, which leads to changes in the development of the hair cycle, miniaturisation of the follicles and inflammation [102].

Early-onset androgenic alopecia is seen as a male equivalent of PCOS [103].

1.1.2 MiRNAs

Micro RNAs (miRNAs) were first described in 1993 [104] and are a group of small non-coding RNA molecules (19-25 nucleotides). Frequently, they control gene expression through silencing the gene by binding to a target messenger RNA (mRNA). This is known as gene regulation. MiRNAs regulate almost all cellular functions. Gene regulation is the cause of many phenotypic variations among individuals and also affects disease processes [105]. MiRNAs are involved in immunity functions, haematopoiesis, neurogenesis, stem cell differentiation, cardiac and skeletal muscle development and are associated with cancer [106]. MiRNAs are found in multiple cell types and blood and can vary in expression, so they rarely fit the ‘on or off’ scheme. Each miRNA can target multiple mRNAs, and one mRNA can be addressed by multiple different miRNAs [107]. 5.300 human genes and approximately 30% of the human genome are regulated by miRNAs [108].

1.1.2.1 Biogenesis of miRNAs

MiRNAs are derived from miRNA genes or intronic regions of coding genes [109]. RNA polymerase II is the major RNA polymerase for miRNA gene transcription into a pre-

cursor miRNA with stem-loop hairpin structure (pri-miRNA) [110]. This pri-miRNA is then processed in the cell nucleus by Drosha (RNase III endonuclease), resulting in the formation of a shorter hairpin-structured pre-miRNA [109]. The pre-miRNA is actively exported from the nucleus to the cytoplasm by exportin 5. Exportin 5 forms a complex with the hairpin structure and Ras- related nuclear protein-guanosine triphosphate (Ran-GTP) [111]. In the cytoplasm, further processing then forms a short double strand miRNA from the pre-miRNA by Dicer (RNase III endonuclease) and transactivator RNA binding protein (TRBP). Subsequently, it is unwound by a helicase and the mature miRNA is generated [105] and incorporated into the argonaute protein (AGO) [112].
Dicer also initiates the formation of rNA-induced silencing complex (RISC), which incorporates the miRNA and is then guided to the target mRNA, where it binds [111].
Besides the canonical pathway described above, the miRNAs can also be produced through two alternative processes (non-canonical pathway). In one pathway, known as mirtron, the short hairpin structure is generated by the spliceosome and a debranching enzyme. In the second pathway, known as short hairpin RNA (shRNA)-derived miRNAs, shRNAs undergo processing by unknown nucleases into pre-miRNAs, which are then processed by Dicer into mature miRNAs [105].

1.1.2.2 Transport of miRNAs

The mechanism of extracellular miRNA transport occurs via two main routes [113]:

- **Active transport via extracellular vesicles:** Exosomes containing miRNAs are created by fusion of the plasma membrane and multivesicular bodies from the perinuclear cytoplasm. It seems that the miRNA content of the extracellular vesicles differs from the miRNA content of the parent cell. This would be a type of active loading or sorting of miRNAs that could differ according to tissue type or metabolic state. It is not entirely clear how extracellular vesicles or exosomes are taken up. Bound extracellular vesicles may directly activate various intracellular pathways, release their content into the cell by membrane fusion, or enter the cell [113].
- **Transport via protein-miRNA complexes:** MiRNAs are also transported in the blood in complexes with proteins: these complexes deliver miRNAs into the

cells. LDL and HDL can transport miRNAs into the bloodstream. After binding to the receptor, they can regulate gene expression in recipient cells [113].

The profile of miRNAs in extracellular vesicles differs significantly from that of miRNA bound to lipoproteins, which represent only a fraction of miRNA transport. MiRNAs can be bound to ribonucleoproteins or nucleolar protein nucleophosmin 1. In addition, miRNAs may leak from broken or damaged cells [113].

1.1.2.3 Functions of miRNAs

MiRNAs regulate almost all cellular functions [105, 106], whereby different mechanisms are involved.

- **As part of a ribonucleoprotein complex:** The miRNA anchors the RISC to its target site, facilitating specific interactions between the miRNA and the target mRNA [107, 111]. Although longer stretches may enhance binding, 7–8 complementary bases are often sufficient for miRNA-dependent targeting. 'Silencing' in RISC can be misunderstood, more often acting to lower or and fine-tune expression rather than silencing [107].
- **Direct suppression of target genes:** MiRNAs not only act on mRNA targets, but can also target various types of non-coding RNAs and even other miRNAs. The miRNA can bind with its seed region (nucleotides 2–8 of the miRNA) or with its central region (bases 9–12) and, depending on the degradation, cause cleavage of the mRNA. If it is a target interaction with high complementarity, this results in robust silencing. In contrast, if it is a target interaction with low complementarity, this leads to translational repression. Other evidence suggests that targets can be degraded even if they do not have extensive complementarity [107].
- **Reduction of target expression:** In addition to the mechanisms already mentioned, miRNAs can reduce target expression through induced decapping, induced deadenylation, altered cap protein binding, diminished ribosome occupancy and sequestration of the mRNA from the translational machinery. These mechanisms are not mutually exclusive and may result in lower mRNA levels or act only by decreasing protein expression [107].

A single miRNA can have opposing functions in different systems. Mostly, miRNAs decrease the expression of targets with miRNA binding sites in the 3'UTR. In some cases, under certain cellular conditions, the repressive function can be overcome, and they even increase the expression of the target [107].

1.1.2.4 Nomenclature

In mature miRNA nomenclature the first 3 letters represent the species name, followed by a hyphen. Thereafter, the prefix 'miR' is added, accompanied by a numerical identifier. The hyphen is added after the prefix to differentiate plant miRNAs from animal miRNAs. Animal pre-miRNAs are named using lower case letters and italics. In contrast, plant pre-miRNAs are named with capital letters. Suffixes are used to indicate closely related or similar mature miRNA sequences derived from different precursors or genomic loci. Sometimes miRNA species are diced from a common precursor. The different mature miRNAs generated by sequential processing from the same precursor are distinguished by an additional numerical suffix after a dot (.). Furthermore, the suffixes -3p and -5p indicate whether the mature miRNA was generated from the 5' or 3' arm of the hairpin precursor, respectively [114].

Species identifier	Dash	Prefix	Dash	Numerical identifier	Suffix	Suffix
hsa	-	miR	-	223		
mtr	-	MIR		5223	b	

Table 1.2: Two examples of naming miRNAs.
First: hsa-miR-223 a humane mature miRNA
Second: mtr-MIR5223b a plant pre-miRNA from medicago truncatula, the barrel-clover;

1.1.2.5 MiRNAs in PCOS

MiRNAs might be implicated in differing PCOS phenotypes through their regulatory roles in key biological processes involved in PCOS pathogenesis. For example a miRNA regulating ovulation could be helpful for distinguishing a women with phenotype A from phenotype C.

Several miRNAs have been linked to PCOS as well as to processes involved in the pathophysiology of PCOS:

- miR 155 is reported to be upregulated in PCOS and seems to have an association with cumulus cell function, oocyte maturation, blastocyst formation and inhibition of T release [115].
- miR 223-3p is reported to be upregulated in PCOS and seems to have no correlation with insulin, HOMA-IT, HOMA-β and T levels, but is associated with IR in human adipose tissue [116].
- miR 29a-5p is reported to be downregulated in PCOS and seems to be associated with follicle growth and insulin [117, 118].
- miR 93 is reported to be upregulated in PCOS and seems to be associated with IR, obesity, and ovarian stimulation [119].
- miR 320 is reported to be downregulated in PCOS and seems to be associated with estradiol, steroidogenesis, insulin signaling and fertilization [120, 121].
- miR 592 is reported to be downregulated in PCOS and seems to be associated with follicular maturation and ovulation [122].
- miR 23a-3p is reported to be downregulated in PCOS and seems to be correlated with BMI [123].
- miR 6767-5p is reported to be downregulated in PCOS and seems to be associated with SHBG, fasting glucose, number of menses per year [124].
- let 7b-3p is reported to be upregulated in PCOS and seems to be related to AMH and to have no correlation with androgens [125].
- miR 1260a is reported to be upregulated in PCOS and seems to be correlated with the free androgen index and have no correlation with androgen levels [125].
- miR 424-5p and miR 18b-5p are reported to be upregulated in PCOS and seem to have no correlation with androgens [125].

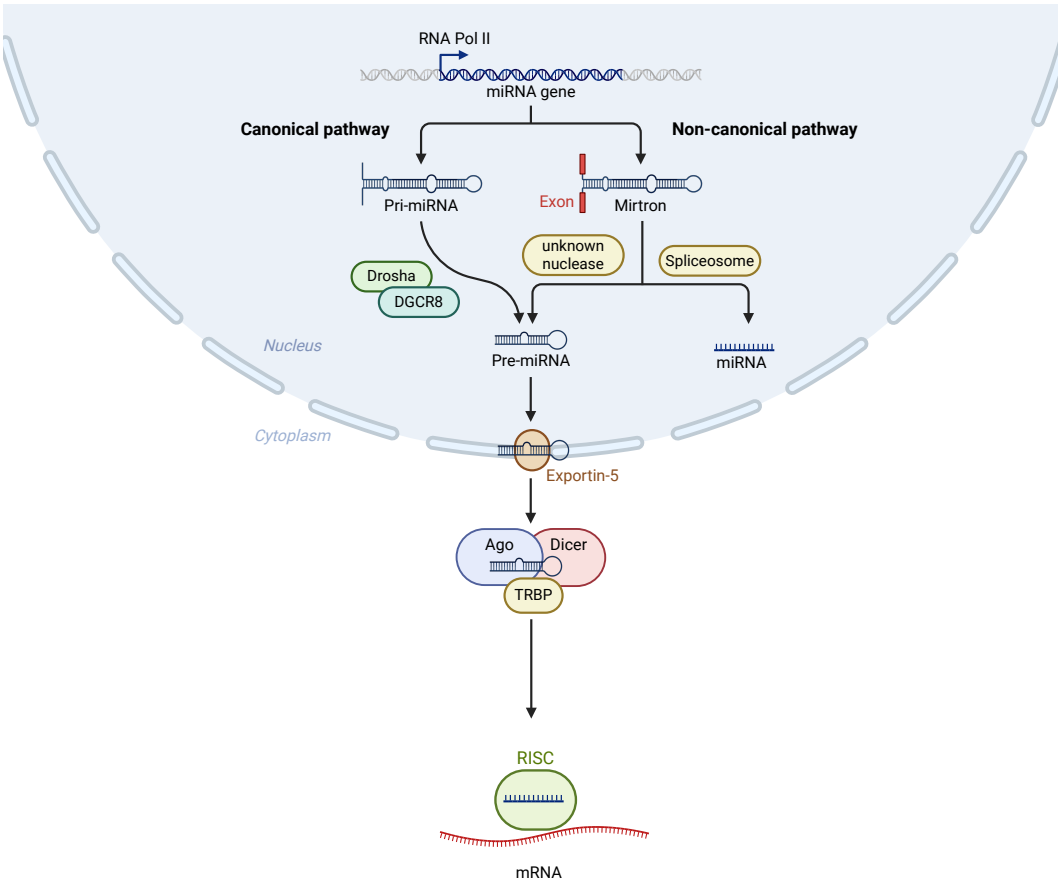


Figure 1.3: RNA polymerase II transcribes miRNA genes into precursor miRNAs (pri-miRNAs) with stem-loop structures. Inside the nucleus, Drosha converts pri-miRNAs into shorter pre-miRNAs. Exportin 5 carries pre-miRNAs to the cytoplasm. There, Dicer and TRBP process them into short double-stranded miRNAs. These mature into single-stranded miRNAs, which combine with argonaute proteins in the RISC. RISC then targets mRNA, controlling gene expression. Alternative pathway: mirtron, formed via the spliceosome and a debranching enzyme, and shRNA-derived miRNAs, produced from shRNAs by unidentified nucleases, further processed into mature miRNAs by Dicer.
Created with BioRender.com

Name:	Reg.	Material	Association	References
miR-155	⇑	Cumulus cell, Serum	Cumulus cell function, oocyte maturation, blastocyst formation, inhibits T release	[115]
miR-223-3p	⇑	Plasma	no correlation with insulin, HOMA-IR, HOMA-β or T levels; ⇑ in IR human adipose tissue	[116]
miR-29a-5p	⇓	Serum	follicle growth, insulin,	[117, 118]
miR-93	⇑	Plasma	IR, obesity, ovarian stimulation	[119]
miR-320	⇓	Serum	estradiol, steroidogenesis, insulin signaliing, fertilization	[120, 121]
miR-592	⇓	Serum	LHCGR (follicular maturation and ovulation)	[122]
miR-23a-3p	⇓	Serum	correlated with BMI	[123]
miR-6767-5p	⇓	Serum	associated with SHBG, fasting glucose, number of menses per year	[124]
let-7b-3p	⇑	Serum	related to AMH, no correlation with androgens	[125]
miR-1260a	⇑	Serum	correlation with FAI, no correlation with androgens	[125]
miR-424-5p	⇑	Serum	no correlation with androgens	[125]
miR-18b-5p	⇑	Serum	no correlation with androgens	[125]

Table 1.3: MiRNAs with reported association with PCOS. These are also the miRNAs chosen for the study. The descensional progress was based on the association and regulation reported in the references shown in the last column.

1.1.2.6 MiRNAs as biomarkers

A big part of research addressing miRNAs as biomarkers investigates oncological disease. In this context, specific miRNA regulation can be indicative for the presence of cancer, its type and its progression. Indeed, numerous studies have shown the diagnostic and prognostic value of circulating miRNAs in various cancer types. Although, before translating this potential into clinical practice, it is important to establish a process for proper validation and standardization [126] see also 2.3.2.

Also, miRNAs can play a role in standardizing of phenotypic expression [127], moreover some circulating miRNAs have been identified as promising prognostic and diagnostic biomarkers for osteoarthritis, again with the key being the identification of robust analytical methods. MiRNA detection can lead to personalized medicine and provide a new approach to the diagnosis and treatment of a disease [128].

In summary, while miRNAs hold great promise as diagnostic biomarkers, their effective utilization in routine clinical diagnosis requires resolving issues related to specificity, reliability, and accessibility of detection methods. In addition to an association of a miRNA with a disease, it is necessary to understand the biological characteristics of these miRNAs in the concerning diseases to improve their future use as biomarkers [129].

1.2 Aim of the study

Research in the field of PCOS is necessary as it is required to advance the understanding of the underlying causes of PCOS and develop more effective treatments, improve diagnostic tools and early detection methods for PCOS as well as identify novel therapeutic approaches. This may be beneficial to prevent long-term health consequences associated with PCOS (see 1.1.1.4). In short, research in PCOS is crucial for improving the lives of millions of women worldwide.

Therefore, it is not surprising that due to the rapidly growing interest in PCOS, the available literature and ongoing research is constantly increasing. Nevertheless, many aspects of PCOS, especially aetiology (see 1.1.1.2) and pathophysiology (see 1.1.1.3), are not yet fully understood. Several studies have already examinated miRNA profiles

in women affected by PCOS [116, 119, 119, 122–125, 130–133], but so far no study has considered the specific PCOS phenotypes.

PCOS and the classification of its phenotype are significant for several reasons: PCOS is a very common disorder affecting many women, PCOS is an enormously complex and heterogeneous disorder with a wide spectrum of symptoms and comorbidities, and these in particular differ relevantly between phenotypes, PCOS may only occur in women (by definition), but the findings of this study may also be relevant to men with symptoms associated with PCOS (see 1.1.1.8).

The aim of this study was to characterize altered miRNAs related to PCOS in serum of women with specific PCOS phenotypes. The potential differences between phenotypes could provide insights into pathophysiological mechanisms that are not yet fully understood.

Furthermore, potential phenotype-specific miRNA biomarkers could be determined with this study.

Sample retrieval proved to be very challenging as it required women who exactly matched the phenotypes under investigation and women who did not fulfil Rotterdam criteria as a control group. The study design can be regarded as a pilot study or search study, which also explains the small sample size.

The primary hypothesis is that women with specific PCOS phenotypes show specific miRNA expression patterns. The secondary hypothesis is that women with hyperandrogenic PCOS phenotypes show specific miRNA expression patterns when compared with non-hyperandrogenic phenotypes.

Chapter 2

Methods and Data

2.1 Population and Sample Collection

The sample shown in figure 2.1 was chosen from the PCOS cohort study. It is a cross-sectional study in which 1598 women aged between 18 and 45 took part. Recruitment started in 2007 and is still ongoing. It is conducted in the endocrine outpatient clinic of the Department of Endocrinology and Diabetology at the Department for Internal Medicine at the Medical University of Graz. Most women were referred to our outpatient clinic by gynaecologists or general practitioners for a detailed PCOS evaluation.

The PCOS cohort study has been approved by the Medical University of Graz (EC 18-066 ex 06-07), for which a written informed consent form was always obtained from the participants prior to recruitment.

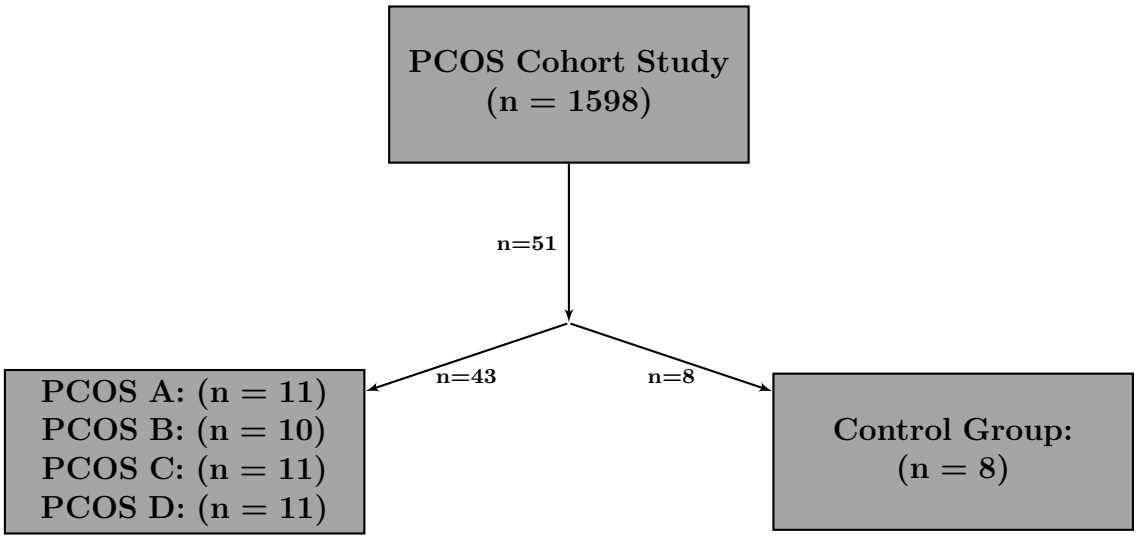


Figure 2.1: The study flowchart for our study shows the initial sample collection out of the PCOS cohort study, from which we selected 51 participants. One path illustrates the selection of the four PCOS groups. The other path shows the control group. This represents the composition of the sample of our pilot study.

PCOS diagnosis was established with the presence of at least two Rotterdam criteria [5].

- **Hyperandrogenism (HA)**:
  - **Clinical HA**: Presence of hirsutism (>4 in the mFGS) [134], acne, alopecia and/or seborrhoea
  - **Biochemical HA**: total T (> 0.77 ng/m), free T (> 3.18 pg/mL), androstenedione (> 3.2 ng/mL) and/or dehydroepiandrosterone sulfate (DHEAS) (> 2.75 ng/mL)
- **Oligo- or anovulation (AO)**: average cycle length > 35 or < 25 days for the past 12 months or a single cycle > 90 days
- **Polycystic ovary morphology (PCOM)**: trans-vaginal ultrasound by qualified gynaecologists

Additionally, other causes for these criteria, such as the Cushing's syndrome, congenital adrenal hyperplasia, androgen-secreting neoplasms or hyperprolactinaemia, were reliably excluded.

The women were categorised into five groups following the table 1.1 (see figure 2.1). Furthermore, we selected BMI and age-matched samples.

At this point, it is relevant to mention that it proved highly challenging to obtain BMI and age-matched samples for all groups, which is why we opted for a small sample size and decided to conduct a pilot study.

All included women had their medical history reviewed by qualified physicians and measurements of height, weight, systolic and diastolic blood pressure, pulse rate, waist and hip circumference and clinical signs of HA were taken. After an overnight fast, blood samples were collected and the following hormone levels were assessed: thyroid-stimulating hormone (TSH), free thyroxine (fT4), free triiodothyronine (fT3), parathyroid hormone (PTH), 25-hydroxy-vitamin D (25OHD), basal cortisol, basal aldosterone and renin, LH, FSH, 17-oestradiol, total T, free T, SHBG, DHEAS, androstenedione, ACTH, human growth hormone (HGH), prolactin, insulin-like growth factor 1 (IGF-1) and routine parameters including a differential blood count, serum creatinine, electrolytes, γ-glutamyl transferase (GGT), aspartate aminotransferase (AST)), alanine aminotransferase (ALT), high-sensitivity C-reactive protein (hsCRP), glycated hemoglobin (HbA1c), total cholesterol, HDL,LDL and triglycerides. Furthermore, patients underwent a 2-h oral glucose tolerance test with 75 mg glucose dissolved in 300 ml water and 4 time points of glucose and insulin measurements (baseline, 30 min, 60 min, 120 min). In addition, medication intake was assessed anamnestically.

This part of the methodology of the PCOS Cohort-study has already been published [74, 135, 136].

2.1.1 Controls

Name		Type
Uni Sp2	Exogenous control	synthetic RNA
Uni Sp4	Exogenous control	synthetic RNA
Uni Sp5	Exogenous control	synthetic RNA
Uni Sp6	Exogenous control	synthetic RNA
miR-484	Endogenous control	miRNA
snU6	Endogenous control	non coding RNA

Table 2.1: These are the RNA molecules used as exogenous and endogenous controls in our study, they where included in specific laboratory steps (see 2.2).

2.2 Laboratory methods

This section describes the laboratory methods applied in a test run conducted by me in June 2023 using kits and manuals from Qiagen [137]. The experiments with the real patient samples were conducted by Reintar Sharmaine in August 2023 using the same protocol and methods.

2.2.1 miRNA Isolation

- **Materials for miRNA Isolation**:
  - Test serum samples (n=8)
  - Qiagen: miRNeasy Serum/Plasma Advanced Kit (ref: 217204) at RT
  - Pipet and tips (RNAsefee/ low binding)
  - 1.5 and 2 ml Eppendorf tubes
  - Centrifuge (Biofuge, heraeus: SNr. 40348274)
  - Vortex
  - Qiagen: RNA Spike-in Kit, for RT (Ref: 339390)

  - RNeasy UCP MinElute spin column
  - RNAse free water
  - Isopropanol
  - 80% ethanol
  - Ice

- **Principles of miRNA Isolation:**

  MiRNA isolation is a process for the extraction and purification of miRNA molecules from biological samples. This process involves the disruption of the cell membrane, the release of the miRNAs from the cells and its subsequent separation from other cellular components to obtain pure miRNA samples for further analysis. The first step is sample lysis, the second is the removal of inhibitors and the third is RNA purification using silica membranes. The miRNeasy Serum/-Plasma Advaced Kit used here was designed specificity for the isolation/purification of total cell-free RNA (primarily miRNA and other small RNAs) from serum or plasma [137].

- **Preparations:**

  - Preparation of Spike-in unit Uni Sp2/4/5
    * Resuspended the Spike in a mix with 80μl nuclease free water
    * 20 minutes on ice for dissolution of the RNA pellet

- **Method of miRNA Isolation:**

  1. Thawed the frozen samples
  2. Prepared mix of 60 μL buffer RPL + 1 μL of prepared spike-in unit Sp2/4/5 (for each sample)
  3. Transferred 200 μL of each serum sample into a 1.5 mL Eppendorf tube
  4. Added 60 μL of the pre-mixed solution (buffer RPL with spike-in Uni Sp2/4/5) to each serum sample.
  5. Vortexed for >5 seconds.
  6. Incubated at room temperature (RT) for 3 minutes.
  7. Added 20 μL of RPP buffer.

8. Vortexed for >20 s.
9. Incubated at RT for 3 minutes
10. Centrifuged at 4100 rpm for 3 minutes at RT to pellet the precipitate.
11. Transferred the supernatant (∼230 μL) to a new reaction tube.
12. Added 1:1 volume of isopropanol:supernatant.
13. Mixed well by vortexing.
14. Transferred each sample to a separate RNeasy UCP minElute column.
15. Centrifuge for 15 s at 3500 rpm and discarded the flow through.
16. Pipetted 700 μL of buffer RWT into every RNeasy UCP MinElute column.
17. Centrifuged for 15 s at 3500 rpm. Discarded the flow through.
18. Pipetted 500 μL of buffer RPE into every RNeasy UCP MinElute column
19. Centrifuged for 25s at 3500 rpm. Discarded the flow through.
20. Pipetted 500 μL of 80% ethanol into every RNeasy UCP MinElute column.
21. Centrifuged for 2 minutes at 3500 rpm. Discarded the flow through and the collection tube.
22. Inserted the RNeasy UCPC MinElute spin column into a new 2 mL collection tube. Opened the lid of the spin column and centrifuged at full speed for 5 minutes to dry the membrane. Discarded the flow through and the collection tube.
23. Placed the RNeasy UCP MinElute spin column into a new 1.5 mL collection tube. Added 20 μL of RNAse-free water directly to the centre of the spin column membrane and incubated for 1 min. Closed the lid, and centrifuged for 1 min at full speed (13000 rpm) to elute the RNA.
24. Labelled the samples
25. Stored the isolated miRNA at -80°C, -2 floor ZMF, labelled as PCOS samples until cDNA synthesis

2.2.2 cDNA Synthesis

- **Materials for cDNA Synthesis**:

- Isolated miRNA samples (stored at -80°C)
- Qiagen: miRCURY LNA RT Kit (Ref: 339340)
- 2 strips of PCR Eppendorf tubes
- Pipet and tips
- Ice
- Qiagen: RNA Spike-in Kit, for RT (Ref: 339390)
- Uni Sp6
- PCR cycler (PEQLAB) VWR brand peqstar N81440 (core facility of molecular biology)
- RNAse free water

- **Principles of cDNA Synthesis:**

  Complementary DNA (cDNA) synthesis is a process in which RNA molecules are converted into cDNA. This cDNA can then be used for various applications such as PCR, gene expression or sequencing.

- **Preparations:**

  - Preparation of Spike-in unit Uni Sp6
    * Resuspended the Spike in mix with 80μl nuclease free water
    * 20 minutes on ice for dissolution of the RNA pellet

- **Method of cDNA Synthesis:**

  1. Thawed frozen miRNA template and 5x miRCURY RT rection buffer on ice.
  2. Thawed RNAse free water at RT
  3. Removed the 10x miRCURY RT enzyme from the freezer.
  4. Mixed the individual solutions by inverting the tubes. Centrifuged briefly to collect residual liquid from the sides of the tubes and then keep on ice.
  5. Prepared the master mix for reverse transcription on ice according to this table. (Note: The final volume is given with an adjusted additional volume of 10% due to possible pipetting errors.)

Component		Final volume
5X miRCURYRT Reaction Buffer	2 μL	28.6 μL
RNase-free water	4.5 μL	64.35 μL
10x miRCURY RT Enzyme Mix	1 μL	14.3 μL
Synthetic RNA spike-ins	0.5 μL	7.15 μL
Template RNA (5ng/μl)	2 μL	28.6 μL
Total reaction volume	10 μL	143 μL

6. Mixed the total volume from the table above.
7. Then, added 8 μL of the reaction mix (prepared in the last step) to each of the Eppendorf strips. Subsequently, 2 μL were added to each miRNA template. Scheme of the strip #1 and #2:

Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
1	2	3	4	5	6	7	8
RT(+)	RT(-)						

8. Mixed and then kept on ice.
9. The RT (+) was prepared with:

Component	Volume
5X miRCURYRT Reaction Buffer	2 μL
RNase-free water	4.5 μL
10x miRCURY RT Enzyme Mix	1 μL
Synthetic RNA spike-ins	0.5 μL
MiRNA pool (1 μL of each miRNA samples)	2 μL
Total reaction volume	10 μL

10. The RT(-) was prepared with:

Component	Volume
5X miRCURYRT reaction buffer	2 μL
RNase-free water	5.5 μL
Synthetic RNA spike-ins	0.5 μL
MiRNA pool (1 μL of each miRNA samples)	2 μL
Total reaction volume	10 μL

11. Put the PCR strips containing the reaction mix into the PCR cycler (programme: ENDO: cDNA QIAGEN miRCURY RT.js)

Step	Time	Temperature
Reverse transcription step	60 min	42°C
Inactivation of reaction	5 min	95°C
Storage	Forever	4°C

12. Stored the cDNA for qPCR

2.2.3 qPCR

- **Materials for qPCR**:
  - Qiagen: miRCURY LNA SYBR Green PCR Kit (Ref: 339347)
  - Primer: Qiagen: miRCURY LNA PCR primers (ref: 339306) lyophilized form
  - 384 well plate (BioRad) and foil cover (BioRad)
  - Gloves
  - Pipettes, multipipettes and tips from Thermofischer, E1 clip tip
  - Centrifuge
  - Vortex
  - Primer sets: miR-155-5p, miR-223-3p, miR-29a-5p, miR-93-5p, miR-320a-3p, miR-592, miR-23a-3p, miR 6767-5p, let-7b-3p, miR-1260a, miR-424-5p, miR-18b-5p, Uni Sp2, Uni Sp4, Uni Sp5, Uni Sp6, miR-484, snU6

  - RNAse free water
  - 1.5 mL Eppendorf tubes

- **Principles of qPCR:**

  This step is based on polymerase chain reaction (PCR), which generally consists of 3 phases that are repeated several times (cycles). The first step is denaturation, in which the cDNA is melted by breaking the hydrogen bonds between the bases, resulting in two single-stranded DNA molecules. The second step is annealing, in which the primers bind to the DNA. A specific primer is used for the specific sequence to be amplified. The DNA polymerase then binds to the primer. In the third step, elongation, the DNA polymerase starts synthesising the new DNA strand.
  With PCR, even a single copy of a specific sequence can theoretically be amplified and thus detected. The ratio between the initial amount of cDNA and the amount of PCR product accumulated in each cycle makes it possible to have a quantitative result. The measurement in qPCR (in every cycle) removes the variability associated with conventional PCR [138].

- **Preparations:**

  - Diluted cDNA (1:30) (every sample + controls)
    * Added 145 μL of RNAse free water
    * Added 5 μL of cDNA and mixed
  - Primer preparation
    * Dissolved lyophilized primer (forward and reverse) in 220 μL of RNAse free water
    * Incubateed for 20 minutes
    * Vortexed
    * Spined down/centrifuge
    * Stored at -20°C

- **Method of qPCR:**

  1. Thawed frozen miRNA template and 5x miRCURY RT rection buffer on ice.

2. Thawed reagents
   - master mix
   - cDNA
   - primer sets
   - RNAse free water
3. Master mix preparation used 1.5 mL Eppendorf tubes (3 Eppendorf tubes, each with a different primer set)

Master mix	μL	x32 wells (in μL)
2x miRCURY SYBR GREEN master	5	192
Primer set	1	38.4
RNAse free water	1	38.4
Total reaction volume: 10 µL	7 + 3 cDNA(1:30)	268.8

4. Added 7 μL of the master mix to each well and added 3 μL of diluted cDNA samples to each well (except for NTC, where we used 3μl of RNAsefree water and IPC, where we used 3μl of pooled cDNA), according to the pipetting scheme.
5. Mixed the reaction and centrifuged/spun the reaction mix briefly in a cooled centrifuge: 500 rpm for 1 min

Plate	miRNAs
1	155-5p; 223-3p; 29a-5p
2	93-5p; 320a-3p; 592
3	23a-3p; 6767-5p; let-7b-3p
4	1260a; 18b-5p; 424-5p
5	Uni Sp 2/4/5
6	Uni Sp6; 484; snU6

6. Used program on the PCR cycler over 40 cycles:

Step	Time	Temperature	Ramp rate
PCR initial heat activation	2 mins	95°C	Maximal / fast mode
2-step cycling			
Denaturation	10 s	95°C	Maximal / fast mode
Combined Annealing/extension	60 s	56°C	Maximal / fast mode
Melting curve analysis		60-95°	

2.3 Data processing and statistical methods

The raw data from qPCR (2.2.3) was processed and evaluated in Microsoft Excel with the aim of obtaining valid data while avoiding unnecessary data loss. Since each qPCR was performed in duplicate we calculated the mean value and the differences between the mean values. Differences not fulfilling the following criteria were marked as invalid [139]. Acceptable Cq ranges under the assumption of a Poisson distribution:

- Cq 25-30: <0,5 Cycle Duplicate-difference
- Cq 30-33: <1 Cycle Duplicate-difference
- Cq >33: <2 Cycle Duplicate-difference

Further, samples with implausible curves were also manually excluded by expert judgement. Also Cq values <7 were evaluated as noise [139]. These criteria led to the following annotations:

- Not Valid: Excluded due to the above criteria (2.3) or curves evaluated as invalid.
- Undetermined: When Cq < 7 (noise [139])
- One duplicate invalid or indeterminate but classified as valid: Included in base of expert opinion after manual validation of Cq and melting curve, even if one duplicate was invalid.
- Valid: Samples where the duplicates had an acceptable difference, the melting curve was plausible and the Cq value was >7.

These criteria were also used for endogenous controls miR-484 and snu6.

The next step was the interplate calibration of each plate using the Cq values from the IPC (Cq of the sample × 22.75 / mean Cq - value of the IPC). Here, 22.75 was a self-selected value that approximately corresponded to the mean value of all IPCS.

We then determined individual cut-off values for each plate by analysing the Cq and melting curves. The cut-off value was set to the value for the last determined plausible curve + 1. Thus, all values that were previously marked as indeterminate were manually set to the cut-off value.

The following step was the normalisation using endogenous controls, where we had three different possibilities:

- miR-484
- snU6: A metabolically stable small RNA that that occurs in the nuclei of eukaryotic cells [140].
- mean value of miR-484 and snU6

We decided to use the mean of miR-484 and snu6 over the other possibilities because the expression of this combination was stable across all the study samples. So we calculated the ΔCq values (ΔCq = interplate calibrated Cq value of the sample - mean interplate calibrated Cq value of miR-484 and snu6). In this step, it was only possible to normalise samples which had valid miR-484 and snu6 Cq values, so the remaining were excluded.

The last two steps of data processing were the calculation of ΔΔCq values (ΔΔCq = ΔCq of the sample - mean ΔCq value of control group) and the fold change values (FC = $2^{-\Delta\Delta Cq}$) [141].

Statistical analysis was performed using SPSS (version 29). Descriptive data were presented as mean ± standard deviation and One-way-ANOVA (+ Dunnet and Tukey Post Hoc) was used to test the paired changes between different subtypes of PCOS and controls. Furthermore, two new groups were formed by calculating the mean of all PCOS groups and the mean of the PCOS groups A, B and C (hyperandrogenic groups) and comparing each with the control group and the non-hyperandrogenic group D, using a T-test or a non parametric Man Whitney U-test if required (chosen significance

level α=.05). For miRNA expression levels, we also used One-way-ANOVA and alternatively Kruskal- Wallis as a non-parametric test if required. For altered miRNAs it was performed an receiver-operating characteristic (ROC) analysis and calculated area under the curve (AUC).

2.3.1 Functional annotation of binding sites

More than one third of human genes seem to be conserved miRNA targets [108]. Binding sites of differentially expressed miRNAs where evaluated by a functional annotation using Target Scan Human 8.0 [142].

This tool provides a powerful statistical analysis of individual miRNA binding sites, with inclusion of the probability of preferentially conserved targeting this is correlating with experimental measurements of repression [143].

2.3.2 Flow of analysis and confounder detection

Figure 2.2 shows a graphical evaluation of the main possible confounders in miRNA analysis. We have carefully analysed the possible confounders and tried to carry out everything as safely as possible. For the study design and sample collection, we had standardized blood sampling and storage, as well as using serum, which is preferable to whole blood. Haemolysis was monitored visually and by measuring the haemolysis index after sample collection.

We also attempted to control some individual variability by using age- and BMI-matched sample groups. This is important as miRNA profiles can also be influenced by obesity. For sample processing, we used a column-based protocol, specifically developed for high-purity miRNA extraction which is more effective and reliable than a TRIzol extraction [144]. For the miRNA measurement, qPCR was used, which is suitable for our study design and more sensitive than microarrays. This step was also controlled with exogenous controls.

The pre-statistical data processing was adapted from de Ronde 2017 [139].

Thus, we normalised with the mean of two endogenous controls: miR-484 and snu6. Then, ratio-based normalisation was performed as well. In our statistical method, we used the Kolmogorov-Smirnov test and the Leven test to check for normal distribution

and variance homogeneity before then conducting a parametric and non-parametric test, respectively, where possible it was performed an ROC analysis and calculated AUC. This confounder analysis was adapted from Tiberio et al. 2015 [126]. After individuating expressed and altered miRNAs, we identified potential target genes using TargetScanHuman 8.0.

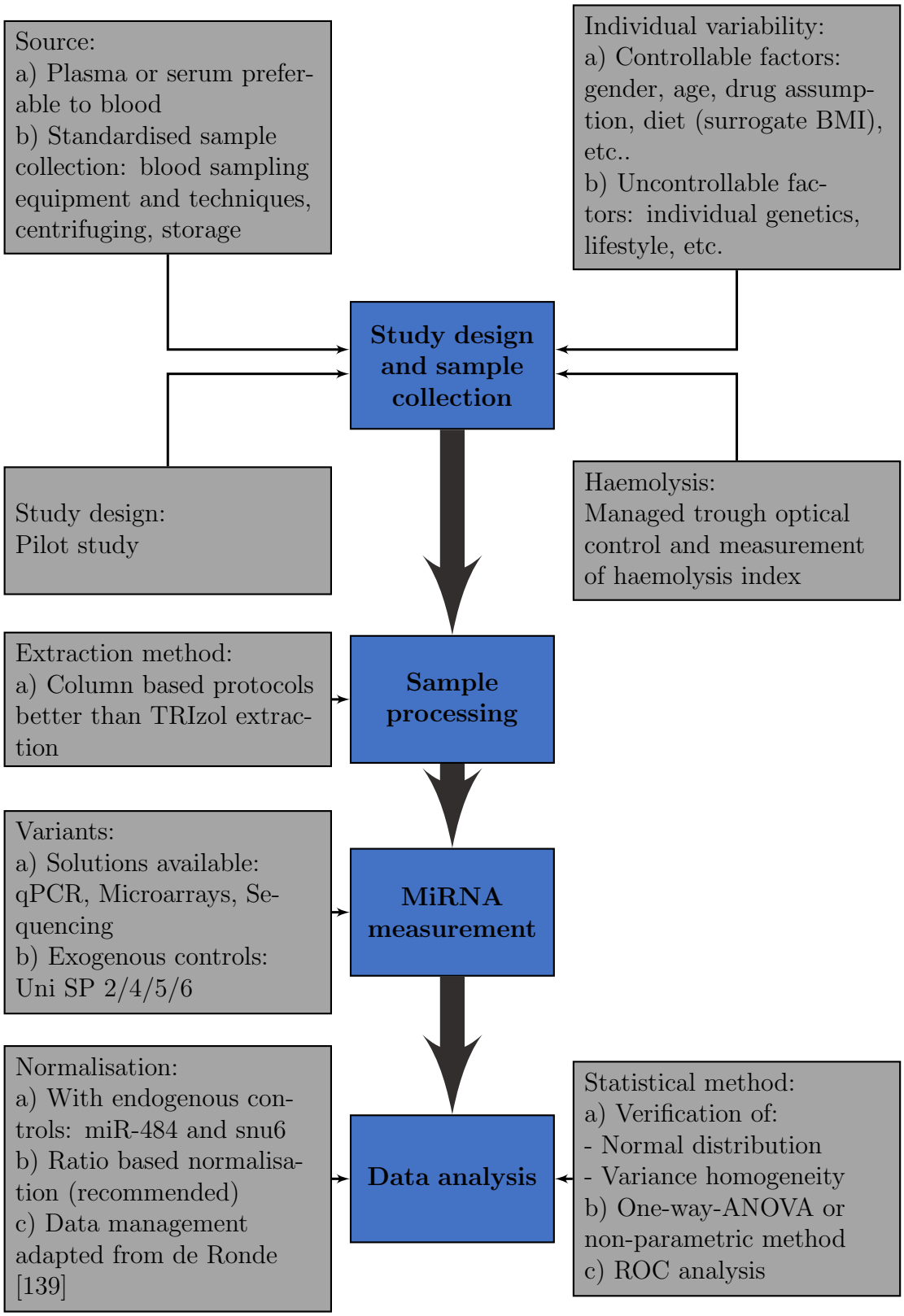


Figure 2.2: Flowchart summarizing the different steps and the main possible confounding factors in miRNA analysis. We have carefully analysed the potential confounders and tried to carry out everything as safely as possible. Adapted from Tiberio et al. 2015 [126].

Chapter 3

Results and Evaluation

3.1 Clinical characteristics

The clinical parameters of the study participants were analysed (see table 3.1). Firstly, a one-way-ANOVA with subsequent post hoc analysis (Dunnet or Tukey) was performed between the five groups, and secondly, the PCOS phenotypes were summarised in one group and compared with the controls using a parametric or non-parametric test. Since the groups were matched in terms of BMI and age, these parameters showed no significant differences. A significant difference between PCOS and the control group was found, when comparing AMH (P<.001), LH (P=.031), androstenedione (P<.001), total (P<.001) and free T (P<.001) levels and hirsutism score (P=.002). In contrast, no significant differences were found in FSH and DHEAS levels when comparing PCOS women with controls. In the subgroup analysis, we found significant differences between PCOS group A and controls in levels of androstenedione (p =.031) and total (p<.001) and free T (P<.001). Between PCOS group B and controls, we identified significant disparities in AMH (P=.043), androstenedione (P<.001) and free T (P=.001) levels. Between PCOS group C and the control group, the differences in DHEAS, (P=.033) total (P=.024) and free T (P=.011) levels and hirsutism (mFGS)(P=.010) however, were found to be statistically significant.

Variable	Control	PCOS A	PCOS B	PCOS C	PCOS D	PCOS
Number	8	11	10	11	11	43
BMI (Kg/m2)	24.53 (±4.50)	25.41 (±6.43)	25.09 (±5.64)	26.278 (±7.07)	27.12 (±8.02)	26.00 (±6.67)
Age (Years)	29.15 (±3.57)	26.42 (±3.86)	29.74 (±3.91)	27.61 (±3.63)	27.51 (±3.05)	27.78 (±3.69)
AMH (ng/mL)	3.95 (±1.85)	12.73 (±7.85)	13.04 (±6.59)^{b}	7.18 (±6.03)	9.96 (±5.63)	10.57 (±6.70)^{d}
LH (mIU/mL)	5.34 (±4.37)	11.32 (±7.14)	15.05 (±11.09)	9.45 (±8.78)	8.70 (±5.01)	11.04 (±8.30)^{c}
FSH (mIU/mL)	5.99 (±2.61)	7.01 (±1.97)	6.66 (±1.65)	5.78 (±3.14)	5.94 (±1.00)	6.34 (±2.09)
LH/FSH Ratio	0.90(±0.52)	1.57 (±0.83)	2.27 (±1.41)	1.90 (±2.10)	1.46 (±0.78)	1.79 (±1.37)^{c}
SHBG (nmol/l)	74.27 (±37.19)	51.93 (±20.65)	66.70 (±43.09)	59.31 (±44.02)	76.55 (±39.01)	63.55 (±37.55)
DHEAS (μg/mL)	1.37 (±0.69)	23.33 (±72.16)	1.82 (±0.76)	2.63 (±0.94)^{a}	1.24 (±0.39)	7.38 (±36.47)
Androst. (ng/mL)	2.33 (±0.58)	3.78 (±1.21)^{a}	4.68 (±1.72)^{a}	3.26 (±1.14)	2.24 (±0.54)	3.46 (±1.46)^{d}
Total T (ng/mL)	0.22 (±0.14)	0.59 (±0.22)^{b}	0.56 (±0.14)^{b}	0.46 (±0.15)^{b}	0.43 (±0.16)	0.51 (±0.03)^{d}
Free T (pg/mL)	1.19 (±0.44)	2.99 (±1.30)^{b}	2.33 (±1.03)	2.64 (±0.85)^{b}	1.67 (±0.57)	2.41 (±1.06)^{d}
Hirsutism (mFGS)	3.25 (±4.5)	6.60 (±5.23)	10.44 (±6.37)	12.18 (±8.10)^{b}	1.11 (±1.45)	7.79 (±7.14)^{d}
PCOM	×	✓	×	✓	✓	✓/×

Table 3.1: These are the results of the descriptive statistical analysis of clinical parameters over the phenotypes and control group
Values are presented as mean ±SD.
a: P value <.05 for the comparison between the PCOS phenotype group vs control using Dunnet Post Hoc;
b: P value <.05 for the comparison between the PCOS phenotype group vs control using Tukey Post Hoc;
c: P value <.05 for the comparison between PCOS vs control using Mann-Whitney-U-test;
d: P value <.05 for the comparison between PCOS vs control using t-test
Androst. = Androstenedione
This table was adapted from Trummer et al. 2024 [1].

3.2 Evaluation of miRNA Expressions

We evaluated the basic expression of our miRNA candidates according to their amount of expression and categorized them into three groups (see 3.1).

- **No expression:** In our study miR-592 and miR-6767-5p were not expressed at all.
- **Expression below detection limit of quantitative measurement:** MiR-155-5p, miR-29a-5p, let 7b-3p and miR-18b-5p, in our samples, showed miRNA concentrations that were too low to be reliably quantified. Although some samples displayed characteristic amplification curves that failed to reach the plateau phase within the predefined 40 cycles, indicating extremely low miRNA expression. For the remaining samples, the initial miRNA concentration was below the detection limit in these qPCRs, thus rendering them undetectable. Among this group of miRNAs, several samples were undetectable: miR-155-5p: n=37 (72.5%), miR-29a-5p: n=30 (58.8%), let-7b-3p: n=24 (47.1%) and miR-18b-5p: n=31 (60.8%). Unfortunately, due to the limited sample size in each group, these miRNAs did not meet the requirements for statistical group comparisons, and such analyses were not conducted.
- **Quantitatively measured expression miRNAs:** MiR-223-3p, miR-93-5p, miR-320a-3p, miR-23a-3p, miR-1260a, and miR-424-5p exhibited valid amplification and melting curves, falling within an acceptable Δ-Cq range of duplicates (see 2.3).

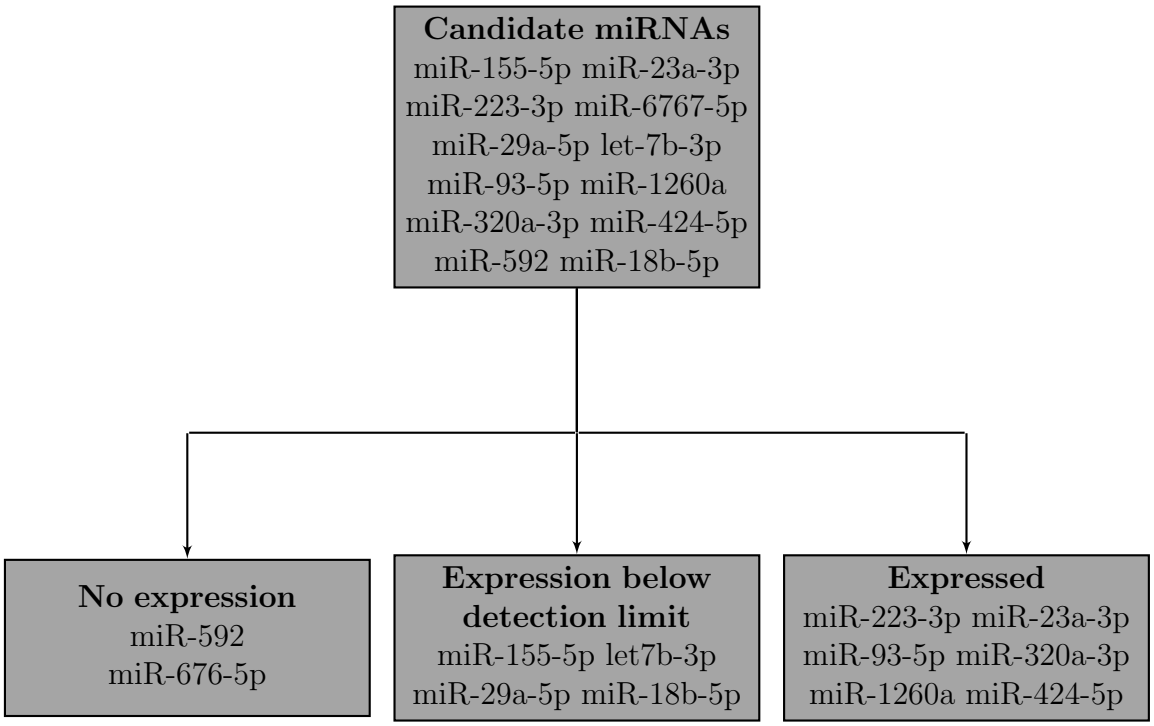


Figure 3.1: Evaluation of the miRNA candidate expression in the study cohort and subsequent categorisation into three groups according to their level of expression in three groups: No expression, expression below the detection limit and quantitatively measured expression.

3.3 MiRNA Expression in PCOS phenotypes

Relevant results were obtained only for the group of expressed miRNAs, where expressions of miR-23a-3p and miR-424-5p were altered when one phenotype was compared to the other phenotypes. MiR-23a-3p was significantly upregulated in PCOS phenotype B in comparison with the other phenotypes. MiR-424-5p was significantly downregulated in PCOS phenotype C in comparison with the other phenotypes (see table 3.2).

MiRNA	Phenotype A	Phenotype B	Phenotype C	Phenotype D	P-value
miR-223-3p	0.51 (±0.20)	0.49 (±0.16)	0.56 (±0.95)	0.55 (±0.19)	.385
miR-93-5p	0.93 (±0.26)	1.36 (±0.37)	0.92 (±0.46)	0.67 (±0.41)	.847
miR-320a-3p	0.51 (±0.20)	0.49 (±0.16)	0.56 (±0.95)	0.55 (±0.19)	.952
miR-23a-3p	1.16 (±0.24)	2.24 (±0.27)	0.95 (±0.45)	0.49 (±0.49)	**.041**
miR-1260a	1.37 (±0.19)	1.43 (±0.57)	1.11 (±1.10)	1.66 (±0.29)	.978
miR-424-5p	1.59 (±0.18)	1.30 (±0.52)	0.24 (±0.36)	1.69 (±0.32)	**.046**

Table 3.2: PCOS phenotype mean miRNA fold change values of the expressed miRNA candidates (group 3) and their P-values. Significance was tested by Kruskal-Wallis test.
This table was adapted from Trummer et al. 2024 [1].

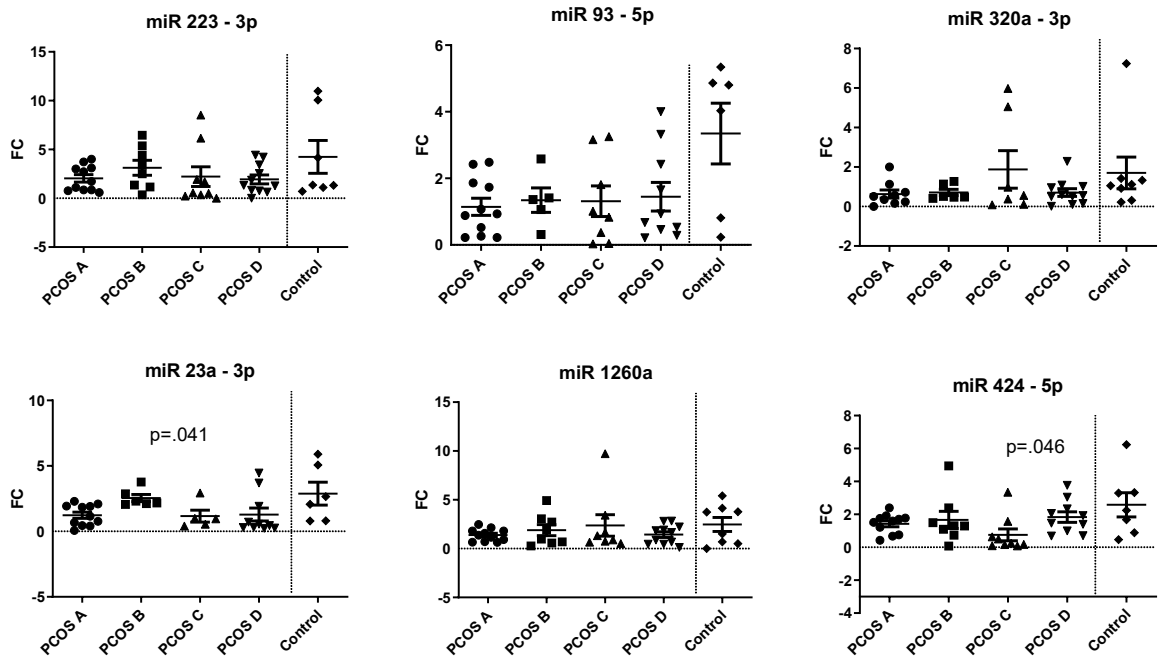
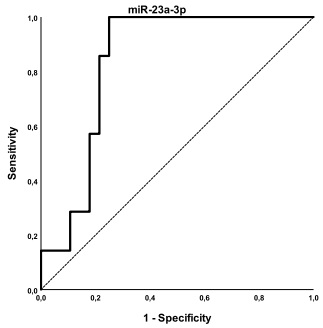
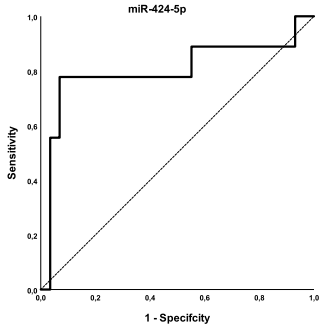


Figure 3.2: Serum miRNA expressions in samples of PCOS phenotypes A,B,C and D. MiRNAs shown: miR-223-3p, miR-93-5p, miR-320a-3p, miR-23a-3p, miR-1260a and miR-424-5p. The data are presented in the form of scatter plots, with each point representing the twofold change as the 2-Cq value of one study sample. P- value was obtained with Kruskal-Wallis test.

The discriminatory potential of miR-23a-3p and miR-424-5p was evaluated through ROC analysis and calculation of the AUC value (Figure 3.3). The results indicate that miR-23a-3p is to be considered as a discriminator to differentiate the PCOS phenotype B from phenotypes A,C and D (AUC= 0.837; 95% confidence interval (CI), 0.706-0.968; P=.006) and miR-424-5p is discriminating PCOS phenotype C from A, B and D (AUC= 0.801; 95% CI, 0.591-1.000; P=.007). The expressions of miR-223-3p, miR-93-5p, miR-320a-3p and miR-1260a were not expressed differently between PCOS phenotype groups (see table 3.2 and figure 3.2).



(a) ROC curve for miR-23a-3p



(b) ROC curve for miR-424-5p

Figure 3.3: The discriminatory potential of investigated miRNAs miR-23a-3p (AUC= 0.837; 95% CI, 0.706-0.968; P=.006) and miR-424-5p (AUC= 0.801; 95% CI, 0.591-1.000; P=.007) displayed in ROC curves.
This figure was adapted from Trummer et al. 2024 [1].

3.4 MiRNA Expression in PCOS vs control

Expression of miR-93-5p is significantly downregulated in women affected by PCOS compared to the control group (P=.042). The fold-change values of miR-93-5p discriminate the PCOS status with a AUC = 0.762; 95% CI, 0.483-1.000; p=0.042. The expression profiles of miR-223, miR-93-5p, miR-320-3p, miR-23a-3p, miR-1260 and miR-424-5p did not show any significant differences between women affected by PCOS and the control group (Table 3.3).

MiRNA	PCOS	Control group	P-value
miR-223-3p	1.71 (±0.32)	1.35 (±1.68)	.335
miR-93-5p	0.93 (±0.18)	4.41 (±0.91)	**.042**
miR-320a-3p	0.52 (±0.23)	1.08 (±0.80)	.122
miR-23a-3p	0.69 (±0.15)	0.80 (±0.42)	.940
miR-1260a	1.35 (±0.27)	2.62 (±0.72)	.434
miR-424-5p	1.16 (±0.12)	0.88 (±0.40)	.928

Table 3.3: Mean miRNA fold-change values of the expressed miRNA candidates and their P-values in women affected by PCOS vs. the control group.
This table was adapted from Trummer et al. 2024 [1].

The discriminatory potential of miR-93-5p was assessed through ROC analysis and the calculation of the AUC value (Figure 3.5). The results indicate that miR-93-5p is

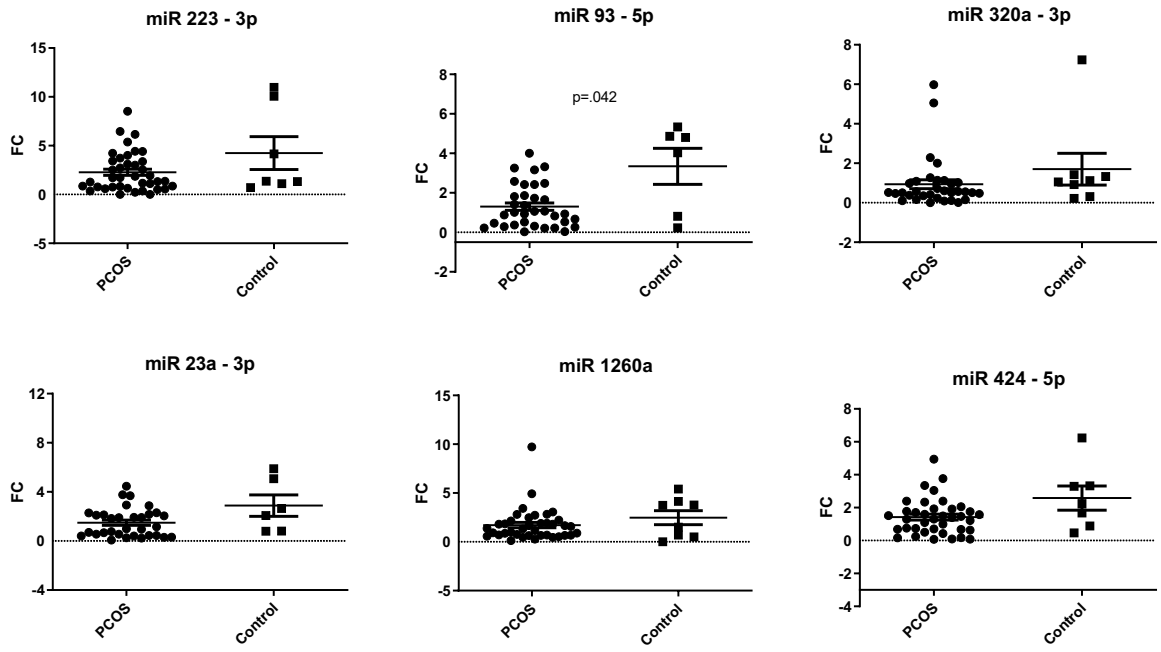
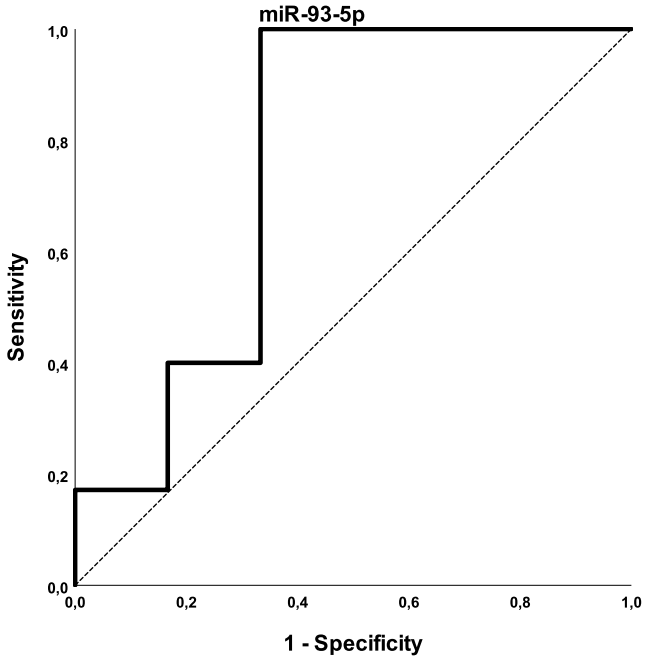


Figure 3.4: Expression of candidate miRNAs in serum of women affected by PCOS and in women of the control group.
MiRNAs shown: miR-223-3p, miR-93-5p, miR-320a-3p, miR-23a-3p, miR-1260a and miR-424-5p. The data are presented in the form of scatter plots, with each dot representing the 2 Cq-fold change in a study sample.

considered as a discriminator to differentiate women affected by PCOS from the control group (AUC= 0.837; 95% CI, 0.706-0.968; P=.006). The systemic expressions of miR-223-3p, miR-23a-3p, miR-320a-3p, miR-1260a, and miR-424-5p were not expressed differentely between women affected by PCOS and the control group (see table 3.3 and figure 3.4).



(a) ROC curve for miR-93-5p

Figure 3.5: The discriminatory potential of investigated miRNA miR-93-5p (AUC= 0.837; 95% CI, 0.706-0.968; P=.006) displayed in ROC curve.
This figure was adapted from Trummer et al. 2024 [1].

3.5 MiRNA Expression in non- vs hyperanodrogenic PCOS phenotypes

Furthermore, the conducted subgroup analysis between hyperandrogenic PCOS phenotypes (A,B,C, n=32) and women affected by PCOS without hyperandrogenism (phenotype D, n=8) showed no significant differences in their expression profiles. The concerning scatter plots are shown in Figure 3.6.

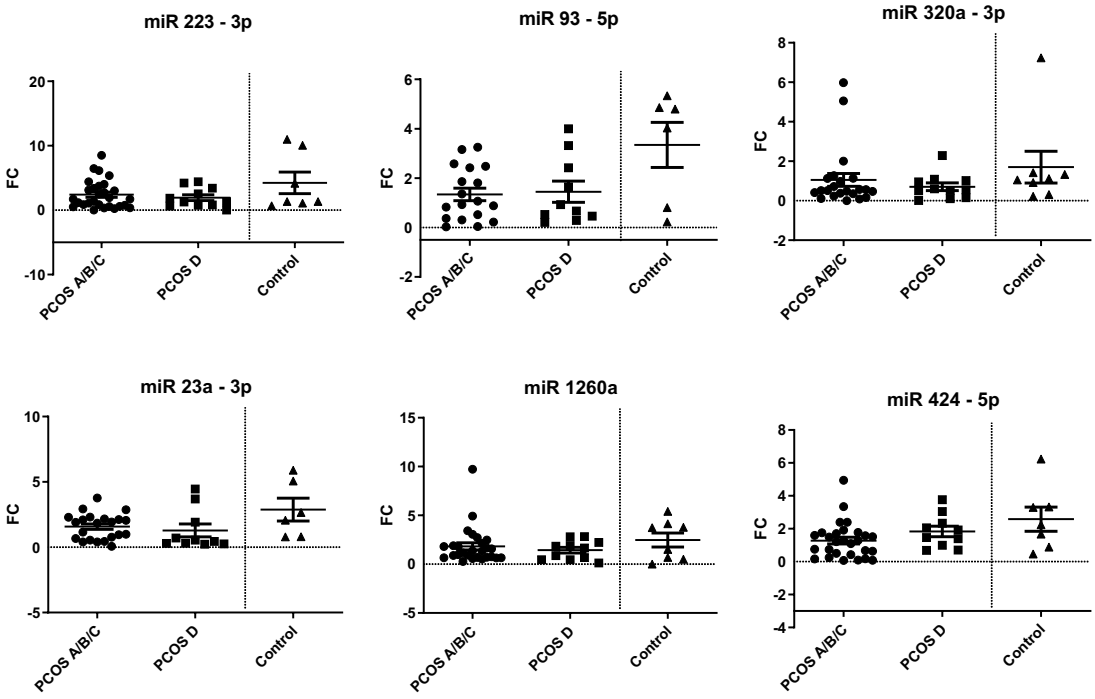


Figure 3.6: Expression of candidate miRNAs in subgroupanalysis of hyperandrogenic phenotypes and women affected by PCOS without signs of hyperandrogenism.
MiRNAs shown: miR-223-3p, miR-93-5p, miR-320a-3p, miR-23a-3p, miR-1260a and miR-424-5p. The data are presented in the form of scatter plots, with each point representing the 2 Cq-fold change in a study sample.

3.6 Potential Binding Site Targets

A functional annotation of the miRNAs (miR-23a-3p, miR-424-5 and miR-93-5p) with altered expressions revealed potential binding sites in genes of molecules implicated in PCOS pathophysiology (IR and carbohydrate metabolism, iron overload, lipid metabolic processes, inflammation, steroid and peptide hormones and fertility) (see section 1.1.1.3 and tables 3.4, 3.5 and 3.6) .

3.6.1 Potential Binding Site Targets for miR-23a-3p

Gene ontologies related to	miR-23a-3p Gene	Function:
IR and carbohydrate metabolism	INPP5A	inositol polyphosphate-5-phosphatase A
	PDK4	pyruvate dehydrogenase kinase 4
	MOGS	mannosyl-oligosaccharide glucosidase
	CHEST3, 7, 10	carbohydrate sulfotransferase 3, 7, 10
Iron overload	TFRC	transferrin receptor
	HSP90B1	heat shock protein 90 beta family member 1
	CYBRD1	cytochrome b reductase 1
	CEMCOX2	cytochrome c oxidase assembly homolog COX15
lipid metabolic process	LRP5	LDL receptor related protein 5
	LIPH	lipase H
	PTGR2	prostaglandin reductase 2
	ALDH1L2	aldehyde dehydrogenase 1 family member L2
Proinflammation/ Inflammation	CCL7	chemokine (C-C motif) ligand 7
	IGSF8	immunoglobulin superfamily, member 8
	CXCL5, 12	chemokine (C-X-C motif) ligand 5, 12
	TNFAIP6	tumor necrosis factor, alpha-induced protein 6
	CRLF3	cytokine receptor-like factor 3
	IL12B	interleukin 12B
	PTGER4	prostaglandin E receptor 4
	IRF 1, 2	interferon regulatory factor 1, 2

Gene ontologies related to	miR-23a-3p Gene	Function:
	SOCS6	suppressor of cytokine signaling 6
	IL6R	interleukin 6 receptor
	HSPH1	heat shock protein family H member 1
	IL11	interleukin 11
	TLR4	toll like receptor 4
hormones and metabolism	STARD3NL	STARD3 N-terminal like
	ESRRG	estrogen-related receptor gamma
	PGRMC2	progesterone receptor membrane component 2
	CYB5R1	cytochrome b5 reductase 1
	THRB	hyroid hormone receptor beta
fertility	NR3C3	
	PGR	progesterone receptor

Table 3.4: Functional annotation of miRNA miR-23a-3p in an exemplary list of potential binding targets associated with signalling pathways important for PCOS.

3.6.2 Potential Binding Site Targets for miR-424-5p

Gene ontologies related to	miR-424-5p Gene	Function:
IR and carbohydrate metabolism	PDK4	pyruvate dehydrogenase kinase 4
	INSR	insulin receptor
	IGF1R	insulin-like growth factor 1 receptor
	CPT1	choline phosphotransferase 1
	NAT8L	N-acetyltransferase 8 like
	INPP5J	inositol polyphosphate-5-phosphatase J

Gene ontologies related to	miR-424-5p Gene	Function:
	PDHB	pyruvate dehydrogenase E1 subunit beta
	CYB561	cytochrome b561
lipid metabolic process	PLA2G15	phospholipase A2 group XV
	ALOX12	arachidonate 12-lipoxygenase,12S type
	EPT1	ethanolaminephospho transferase 1 (CDP-ethanolamine-specific)
	ACACB	acetyl-CoA carboxylase beta
	LRP2	LDL receptor related protein 2
	DHDDS	dehydrodolichyl diphosphate synthase subunit
Proinflammation/ Inflammation	TNFSF13B	TNF superfamily member 13b
	N4BP1	NEDD4 binding protein 1
	ENTPD7	ectonucleoside triphosphate diphosphohydrolase 7
	SOCS6	suppressor of cytokine signaling 6
	CXCl10	chemokine (C-X-C motif) ligand 10
	IRAK2	interleukin 1 receptor associated kinase 2
	SOCS5	suppressor of cytokine signaling 5
	IL2RB	interleukin 2 receptor, beta
	CX3CL1	C-X3-C motif chemokine ligand 1
hormones and metabolism	PTH	parathyroid hormone
	GHR	growth hormone receptor
	ADRB2	adrenoceptor beta 2
fertility	OEEP	oocyte exression protein

Table 3.5: Functional annotation of miRNA miR-424-5p in an exemplary list of potential binding targets associated with signalling pathways important for PCOS.

3.6.3 Potential Binding Site Targets for miR-93-5p

Gene ontologies related to	miR-93-5p Gene	Function:
IR and carbohydrate metabolism	ST8SIA2	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 2
	SLC48A1	solute carrier family 48 member 1
	CHST7	carbohydrate sulfotransferase 7
	SESN3	sestrin 3
Iron overload	CYBRD1	cytochrome b reductase 1
lipid metabolic process	VLDR	very low density lipoprotein receptor
	MSMO1	methylsterol monooxygenase 1
	CYP26B1	cytochrome P450, family 26, subfamily B, polypeptide 1
	SCD5	stearoyl-CoA desaturase 5
	IPMK	inositol polyphosphate multikinase
	LRP8	LDL receptor related protein 8
	ACSF2	acyl-CoA synthetase family member 2
Proinflammation/ Inflammation	IL8	interleukin 8
	ZBTB7A	zinc finger and BTB domain containing 7A
	CCL1	chemokine (C-C motif) ligand 1
	IRF9	interferon regulatory factor 9
	TNFRSF21	tumor necrosis factor receptor superfamily, member 21
	CXCL14	chemokine (C-X-C motif) ligand 14
	CMKLR1	chemokine-like receptor 1
	ITGB8	integrin, beta 8
	CD274	CD274 molecule
	IRF1	interferon regulatory factor 1

Gene ontologies related to	miR-93-5p Gene	Function:
	TNFSF11	tumor necrosis factor (ligand) superfamily, member 11
	SMAD7	SMAD family member 7
	TNFAIP1	tumor necrosis factor, alpha-induced protein 1 (endothelial)
hormones and metabolism	THRA and B	thyroid hormone receptor, alpha and beta
	ESR1	estrogen receptor1
fertility	MMP2	matrix metallopeptidase 2

Table 3.6: Functional annotation of miRNA miR-93-5p in an exemplary list of potential binding targets associated with signalling pathways important for PCOS.

Chapter 4

Discussion

4.1 Discussion of the results

A total of 51 participants were involved in this preliminary study, 11 of whom were in the PCOS phenotype group A, C and D, 10 in group B and 8 in the control group. Compared to the control group, women with PCOS displayed significantly higher levels of various hormones, including AMH, LH, and T, along with elevated hirsutism scores. Phenotypes A and B exhibited notably higher androgen levels, phenotype C showed higher DHEAS and T levels, accompanied by a higher hirsutism score. Phenotype D showed no significant difference from the control group.

Among the miRNA candidates, some showed very low or no expression. However, miR-223-3p, miR-93-5p, miR-320a-3p, miR-23a-3p, miR-1260a and miR-424-5p were expressed. In particular, miR-23a-3p was upregulated in phenotype B, while miR-424-5p was downregulated in phenotype C compared to the other phenotypes. MiR-93-5p expression was lower in the PCOS group than in the control group; however, when corrected for multiple testing, significance was not maintained.

The potential binding sites of these three miRNAs were located in genes of signalling pathways important for PCOS including insulin resistance, lipid metabolism, inflammation, and fertility-related-genes.

Overall, this study revealed differential hormone and miRNA expressions across PCOS phenotypes and potential implications for pathways linked to PCOS characteristics.

Women affected by PCOS, especially in phenotypes A and B, displayed significantly elevated levels of androgens such as T derivatives. Phenotype C showed higher DHEAS and T levels. These hormonal imbalances are a known characteristic of PCOS and contribute to its clinical phenotype. In this context, AMH, LH, and LH/FSH ratio were also notably higher in PCOS groups compared to controls. No difference was found for other variables where a significant variation would have been expected. This

and the increased standard distribution for some values in table 3.1 can be explained by the heterogeneity of PCOS. For example, in PCOS HA is defined by clinical or biochemical HA, so that a woman belonging to phenotype group A can also have normal androgen levels if presenting clinical HA as elevated mFGS. This also makes clear why phenotype D did not significantly differ from the control group.

Some miRNAs were either not expressed or showed only very low expression levels at or below the detection limit in our study group. This can potentially be explained by looking at previous literature. Here it is evident that different tissues such as follicular fluid, granulosa cells, cumulus cells, serum, and plasma were used in publications. Compared to Butler et al. [125], who reported altered expression of miR-1260, and miR-424-5p in plasma of women with PCOS, we could not observe these alterations in serum. Serum and plasma differ in their content of miRNA derived from different blood cells [145, 146] which may be reason for this incongruent finding. Interethnic expression differences between Asian (Xiong et al. [123]) and Caucasian (present study) cohorts could be a further reason. The quality of results in such studies generally differ by the pre-analytical steps, such as blood drawing and serum or plasma preparation. Additionally, the very low amounts of miRNAs, high levels of potential inhibitors, individual biological variances (diet, stress, exercise, age) as well as normalization strategies determine the variating results of different miRNA studies [147].

This is the first study to provide data on miRNAs examined in the four Rotterdam PCOS phenotypes.

However, miR-23a-3p was upregulated in phenotype B, while miR-424-5p was downregulated in phenotype C and miR-93-5p showed lower expression in PCOS compared to control group, suggesting potential roles in distinguishing between these groups. AUCs of these miRNAs were higher than 0.706, this indicates a “fair” test with sufficient balance between sensitivity and specificity for the discrimination of phenotype B for miR-23a-3p and phenotype C for miR-424-5p [148] (see figures 3.3, 3.5).

The potential binding sites of the identified miRNAs are closely linked to specific signalling pathways associated with IR, lipid metabolism, inflammation, and fertility-related genes. This emphasises the value of examining these miRNAs in deciphering the pathophysiology of PCOS.

MiR-23a-3p and miR-424-5p exhibited promise in distinguishing between specific PCOS phenotypes, suggesting their potential as biomarkers for phenotype classifica-

tion. In particular, miR-23a-3p and miR-424-5p could be potential biomarkers that can be able to distinguish between different PCOS phenotypes and could further be relevant for a phenotype classification. However, further in-depth research is essential to validate these findings.

The potential miRNA binding sites with their associated signalling pathways may help to elucidate the potential mechanisms underlying PCOS characteristics.

By understanding the underlying mechanisms of PCOS traits through the identification of binding sites and their correlation with signalling pathways, we could pave the way for tailored therapies targeting hormonal imbalances and specific pathways associated with each phenotype.

These newly discovered miRNAs have the potential to serve as valuable diagnostic or prognostic markers for the accurate classification of PCOS.

4.2 Limitations

The miRNAs analysed displayed low expression levels in serum, thereby limiting the detection of small effects. Besides, due to the sample size of our study, the generalisability of the results towards larger and more diverse populations is restricted. Given some constraints in in sample acquisition, however, we were required to design this research as a pilot study involving 51 participants, so the other limitations were assessed in this regard.

A key aspect worth noting is that our study is cross-sectional, thus only capturing a momentary snapshot of hormonal and miRNA profiles at a given time point. This limitation restricts our ability to attribute causality or track variations longitudinally in the development of the PCOS phenotype.

Even considering the confounding analysis performed (see 2.2), the influence of unknown confounding variables that may impact the observed hormonal and miRNA variations cannot be excluded.

The strengths of our study consist in performing biochemical characterisations in all PCOS patients, sample matching based on age and BMI, and applying a standardised data processing pipeline to achieve validity and reproducibility.

4.3 Outlook

Further research is essential to thoroughly explore the topic. A deeper understanding of the diverse miRNA profiles across the different phenotypes of PCOS could be the base for more personalised and targeted treatment options. Measures specifically tailored to these profiles could lead to the development of new treatment modalities or improve the efficacy of existing methods.

The identified miRNAs, namely miR-23a-3p and miR-424-5p, hold the potential to serve as biomarker for distinguishing between different PCOS phenotypes. Furthermore, the investigation and validation of these results could facilitate the development of more precise tools for categorising phenotypes.

The following directions could be considered for further research:

- Conduct longitudinal studies to observe the evolution of hormonal and miRNA changes within each PCOS phenotype. This will provide insight into the progression of the condition and how these potential biomarkers fluctuate or stabilise with age or different stages of the syndrome.
- Investigate the pathways regulated by miR-23a-3p and miR-424-5p in different PCOS phenotypes. Understanding their specific targets and the downstream effects may reveal fundamental mechanisms underlying phenotypic variations.
- Verify the functions of the identified miRNAs in the control of hormonal pathways. This could include altering expression in vitro or using animal models and observe subsequent effects on hormone levels and phenotypic traits.

Also, this study was published on 11 March 2024 in the „International Journal of Molecular Sciences“ with the title: „Serum Expression of miR-23a-3p and miR-424-5p Indicate Specific Polycystic Ovary Syndrome Phenotypes: A Pilot Study“ [1].

4.4 Conclusion

In summary, these findings contribute to a deeper understanding of the heterogeneous nature of PCOS by highlighting distinct hormonal and miRNA profiles across different phenotypes, which could have implications for diagnostics, treatment, and further

research into targeted therapeutic approaches. Thus, our data provides a first insight into systemic miRNA alterations in PCOS phenotypes and emphasises the potential of miRNAs as clinical biomarkers.

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