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The Search for the Causes of Common Hyperandrogenism, 1965 to circa 2015

Short title: Seeking the causes of premature adrenarche & PCOS

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23 **Abstract**

24 From 1965-2015, immense strides were made into understanding the mechanisms
25 underlying the common androgen excess disorders, premature adrenarche and
26 polycystic ovary syndrome (PCOS). The author reviews the critical discoveries of
27 this era from his perspective investigating these disorders, commencing with his
28 early discoveries of the unique pattern of plasma androgens in premature
29 adrenarche and the elevation of an index of the plasma free testosterone
30 concentration in most hirsute women. The molecular genetic basis, though not the
31 developmental biologic basis, for adrenarche is now known and 11-oxytestosterones
32 shown to be major bioactive adrenal androgens. The evolution of the lines of
33 research into the pathogenesis of PCOS is historically traced: research milestones
34 are cited in the areas of neuroendocrinology; insulin resistance, hyperinsulinism,
35 type 2 diabetes mellitus; folliculogenesis; androgen secretion; obesity; phenotyping,
36 prenatal androgenization, epigenetics, and complex genetics. Large scale genome-
37 wide association studies led to the 2014 discovery of an unsuspected steroidogenic
38 regulator *DENND1A* (differentially expressed in normal and neoplastic
39 development). The splice variant *DENND1A.V2* is constitutively overexpressed in
40 PCOS theca cells in long-term culture and accounts for their PCOS-like phenotype.
41 The genetics are complex, however: *DENND1A* intronic variant copy number is
42 related to phenotype severity, and recent data indicates that rare variants in a
43 *DENND1A* regulatory network and other genes are related to PCOS. Obesity
44 exacerbates PCOS manifestations via insulin resistance and pro-inflammatory
45 cytokine excess; excess adipose tissue also forms testosterone. Polycystic ovaries in
46 40% of apparently normal women lie on the PCOS functional spectrum. Much
47 remains to be learned.

49

50 **Abbreviations**

51 Competitive protein binding (CPB),

52 Congenital adrenal hyperplasia (CAH)

53 Cytochrome P450c17 gene (*CYP17A1*),

54 Dehydroepiandrosterone (DHEA) and DHEA sulfate (DHEAS),

55 Differentially expressed in normal and neoplastic development protein, isoform 1A,

56 variant 2 (DENND1A.V2),

57 Functional ovarian hyperandrogenism (FOH),

58 Genome-wide association studies (GWAS),

59 Hydroxysteroid dehydrogenase (HSD),

60 17-ketosteroids (17KS)

61 Polycystic ovary morphology (PCOM, ultrasonographically defined),

62 Polycystic ovary syndrome (PCOS),

63 Sex hormone binding globulin (SHBG),

64 Type 2 diabetes mellitus (T2DM)

65 Zona reticularis of the adrenal cortex (ZR)

66

67

68 **1 Introduction**

69 The common hyperandrogenic disorders of children and adult women, premature
70 adrenarche and polycystic ovary syndrome (PCOS) were recognized 1935-1952, but
71 our understanding of their pathogenesis dates from the mid-1960s. At that time,
72 measurements of hormones in blood were introduced, and these were followed by
73 an accelerating pace of advances in biochemical and molecular genetics that
74 permitted increasingly sophisticated understanding of the endocrinology of these
75 disorders. However, the broad diversity of findings led to disparate interpretations
76 that have lingered past their time. It is the purpose of this historical review to
77 illustrate from a personal perspective the evolution of the different paths of
78 discovery from 1965-2015 that led to our current understanding of the
79 pathogenesis of these disorders.

80

81 Premature adrenarche accounts for $\geq 90\%$ of the cases of isolated premature pubic
82 hair development (premature pubarche), i.e., before 8 years of age in girls and 9
83 years in boys (1). Premature pubarche occurs in 3-5% of children, and is a common
84 finding ($>7.5\%$) in girls attending pediatric clinics (2). PCOS prevalence is 5-10%
85 among reproductive age women (3). Thus, both are much more common than the
86 virilizing disorders that may resemble them in their presentation. The most common
87 of the latter is 21-hydroxylase deficient congenital adrenal hyperplasia (CAH): the
88 prevalence of the nonclassic form of the disorder, which presents with androgen
89 excess in early childhood, adolescence, or adulthood, is 1:1000-1:2000, 10-fold
90 greater than that of the classic form of the disorder which also presents in early
91 childhood in boys, but neonatally in girls (4).

92

93 This history is my perspective on how the important lines of research into the
94 causes of premature adrenarche and PCOS evolved during my career investigating
95 these disorders 1965-2015, and it concludes with a look forward to how these relate to
96 current issues in research, with the help of systematic PubMed searches on these
97 topics. I begin with the foundational discoveries that paved the way to the starting
98 gate, so to speak. The main section of this review then commences when I entered
99 the field, developing assays for androgens in blood and applying them to studies of
100 premature adrenarche and hirsutism. The text is organized around the seminal
101 discoveries (**Table 1-2**) and the research that each spawned. There have been
102 myriad basic science advances during this period, but only those most directly
103 related to the pathogenesis of premature adrenarche and PCOS are covered here.
104 The history includes my personal history of how I entered clinical research from a
105 background in clinical medicine. But this is mostly a story of ideas, of how medical
106 puzzles have been (as yet incompletely) solved when the nature of the pieces of the
107 mechanism is not known and then, as these are revealed one-by-one, how they fit
108 together is only gradually discovered.

109

110 **2 Foundational observations in female sex steroid endocrinology**

111 **2.1 The adrenal zona reticularis and adrenarche**

112 An authoritative review of the history and function of the adrenal gland attributes
113 the discovery of the adrenal gland to the Greco-Roman physician Galen (ca 130-
114 201AD) (5). The foundational observations of the adrenal zona reticularis (ZR), now
115 known to be the major source of adrenal androgens, date to 1866, when Dr Julius
116 Arnold, according to Dr. Joseph Marshall Flint described in 1900 “the generally
117 accepted nomenclature of the (adrenal) cortex...into three layers...named... from

the arrangement of the blood vessels and connective tissue” (6, 7). Flint provided illustrative figures of this zonation that make clear the reticular nature of the ZR scaffolding (**Fig. 1**) (6).

The term “adrenarche” was coined by the trailblazing Massachusetts General Hospital (MGH) physician Fuller Albright in 1942 to explain the growth of pubic and axillary hair in girls with probable gonadal dysgenesis who lacked breast and uterus development (8). These children excreted more 17-ketosteroids (17-KS) than expected in adrenal insufficiency, though subnormal, which he attributed it to the production of a testosterone-like, nitrogen-retaining (“N”) hormone by the adrenal gland (8, 9).

Dr. Nathan Talbot’s MGH group, although not Albright collaborators (5) adapted Albright’s concept to the condition of isolated premature sexual hair development, attributing it to unusually early production of 17-KS and terming the condition “precocious adrenarche” (10). Lawson Wilkins, MD at Johns Hopkins Hospital disputed Talbot’s conclusion. He termed the condition “premature pubarche” irrespective of mild elevation of 17-KS output, which Wilkins thought “...may be due to minor variations in technic (sic)” (11). Today “premature pubarche” has come to refer to the onset of sexual hair development, irrespective of cause.

2.2 The ovary and its hormones

The ovaries were known to Aristotle (384-322 BC) as the source of sexual behavior and fertility: he is quoted thusly: “the ovaries of sows are excised with a view to

143 quenching their sexual appetites and...female camels are mutilated to prevent their
144 being got with young" (12). Soranus provided the first written description of the
145 ovaries as "didymi (paired organs)...attached to the outside of the uterus, near the
146 isthmus, one on each side"(12).

147

148 The first accurate picture of the female reproductive system appeared in *De Humani*
149 *Corporis Fabrica* (1553), prepared by Andreas Vesalius of Brussels while he was a
150 Professor at the University of Padua (12, 13). Vesalius' figure of the "female testes"
151 is recognized as the first description the ovarian follicles (**Fig. 2**) (14)—this also
152 appears to be the first depiction of polycystic ovaries! Gabrielle Falloppio, a
153 successor at Padua, provided an appreciatively corrected commentary on Vesalius'
154 work, *Observationes anatomicae*, in 1562 (13). In 1671 the Italian anatomist
155 Marcello Malpighi concluded that the "female testes" of the cow were ovaries and
156 named the corpora lutea (15). In 1672 the Dutch physician Regnier de Graaf
157 published observations on his dissections of the human "female testes", also
158 terming them ovaries after showing that they contained vesicles (now termed antral
159 or "Graafian" follicles) that he considered to be eggs by analogy to birds' eggs (15).
160 To him is also attributed the first detailed description of the human corpus luteum
161 and its association with pregnancy (16). The discovery of the mammalian oocyte
162 was reported in 1827 by the embryologist Carl Ernst von Baer (17).

163

164 Turning to hormones-- The first associations of hirsutism and infertility have been
165 traced to descriptions by the Greek physician Hippocrates (ca. 400 BC) (18, 19).
166 Soranus of Ephesus (AD ca. 50 AD) later described this as well. Otherwise only
167 sporadic descriptions of this association are to be found during the middle ages (18,

19). During the 1800s, such reports increased and reports of associations with menstrual disorders and/or “sclerocystic” or “microcystic” ovaries appeared; however, the association of the ovaries with the other two abnormalities was described only in isolated cases (18).

The discovery of the hormonal function of the ovaries began in 1921 with the detailed observations of the sow estrus cycle by George W Corner, MD (20). His studies showed the cyclic chain of events beginning with rupture of the ovarian follicle, followed by the organization of the corpus luteum from successive vascular invasion of the ruptured follicle and fusion of its granulosa and theca cells. He further demonstrated that the characteristic periodic uterine mucosal proliferation was related to corpus luteum development (16, 20). He followed with the description of the rhesus monkey’s menstrual cycle (21). Corner and others then embarked on what proved to be an era of discovery of the steroidal nature of sex hormones.

In 1923 Edgar Allen, PhD and Edward A Doisy, PhD reported that the ovarian follicles contained a feminizing hormone: partially purified extract of follicular fluid from sow ovaries injected into oophorectomized rodents caused vaginal cornification (Allen-Doisy bioassay) (22). Female hormone was first isolated in 1929 from human pregnancy urine by the Doisy and Adolph Butenandt groups (23-25): this was estrone, initially named folliculin, theelin, and progynin (**Fig. 3**). The following year Doisy and Guy Frederic Marrian, PhD independently crystallized estriol (initially termed theelol by the Doisy group) from pregnancy urine, which later proved to almost entirely be a product of the fetal adrenal-placental unit (25,

193 26). In 1936, the estrogenic hormone initially reported in sow ovaries was
194 crystallized from follicular fluid aspirated from 4 tons of sow ovaries in the Doisy
195 laboratory and was found to be estradiol (initially “dihydrotheelin”) (25, 27).
196
197 In 1929, Corner and Willard M Allen demonstrated that the corpus luteum produced
198 a hormone that supported the proliferation of the uterine mucosa (Corner-Allen test)
199 and pregnancy in the castrated rabbit, and in 1930 they isolated the active
200 substance from alcohol extracts of sow corpora lutea (16, 21). From such extracts,
201 Allen and Oskar Wintersteiner, PhD in 1934 prepared a crystalline progestin (28),
202 almost simultaneously with 3 other groups: that of Butenandt, which determined
203 the structure, Slotto and Ruschig; and Hartmann and Wettstein (29, 30). By mutual
204 consent the compound was named progesterone (31).
205
206 The discovery of androgens is ascribed to Arnold Berthold’s 1847 report that testes
207 produced a circulating masculinizing substance: cock’s comb regression after
208 castration was reversed by transplanting testes into the abdominal cavity (32). This
209 report was overshadowed by Brown-Sequard’s 1889 infamous claim that aqueous
210 testicular extracts rejuvenated him (steroids are poorly water-soluble) (33). The first
211 naturally occurring androgen, androsterone, was isolated from policemen’s urine by
212 Butenandt and Tscherning in 1931, the second—dehydroepiandrosterone (DHEA)--
213 was isolated similarly by the same group in 1934 (25). Testosterone was isolated
214 the following year from bull testes by Ernst Laqueur and associates (funded by
215 Organon pharmaceuticals) (34). Within the year, testosterone was chemically
216 synthesized from cholesterol by Butenandt and Hanisch (Schering) and by Leopold
217 Ruzicka and A. Wettstein (Organon) (35, 36). (Edward C Kendall’s group isolated

218 and identified cortisone as a steroid the same year) (37).) Formation of testosterone
219 and androstenedione from ^3H -17-hydroxyprogesterone and ^{14}C -progesterone by a
220 normal human ovary homogenate was documented in 1961 by Ralph Dorfman,
221 PhD's group (38), and secretion of androgens by the ovary was documented in 1966
222 when specific assays for androgens in blood were developed, as reviewed below.

223

224 The Nobel Prize in Physiology or Medicine was awarded to Doisy in 1943, not for his
225 discovery of estrone or estradiol, but for his "discovery of the chemical nature of
226 vitamin K". Butenandt and Ruzicka were awarded the 1939 Nobel Prize in Chemistry
227 for the synthesis of testosterone but prevented from accepting it by the German
228 Nazi government (39), though Ruzicka, who did not participate in the Nazi war
229 effort, gave his Nobel lecture after World War II (5).

230

231 The necessity of the anterior pituitary gland for gonadal, adrenocortical and thyroid
232 development was demonstrated in 1926 by Philip E. Smith by means of
233 transsphenoidal ablation and transplantation of the pituitary of the rat (40, 41). Just
234 five years later, the first definite evidence that two pituitary gonadotropins are
235 required for ovarian development was obtained by Frederick Hisaw and H.L. Fevold:
236 they prepared one purified anterior pituitary fraction that stimulated ovarian
237 follicular growth and another that luteinized the follicles (42). In 1941 they
238 demonstrated that the two sheep gonadotropins were synergistic in stimulating
239 ovarian estrogen secretion (43), and in 1942 Roy Greep and colleagues reported
240 that while swine FSH stimulated follicular growth in hypophysectomized rats, it did
241 not stimulate estrogen secretion until highly purified swine LH, which alone
242 stimulated growth of theca cells but did not stimulate estrogen secretion, was

added (44). Replication of these findings required highly purified gonadotropin preparations (45),

The first evidence for a two-cell theory for follicular estrogen production was obtained by Falck in 1959 when, using an ocular explant bioassay system, he reported that rat follicle estrogen biosynthesis required both granulosa and theca-interstitial cell aggregates (46). He later reported, using a similar bioassay system, that theca-interstitial cells produced only androgen (47).

Drs Irving Stein and Michael Leventhal in 1935 were the first to report a series of cases (n=7) with the triad of polycystic ovaries (**Fig. 4**), amenorrhea, and hirsutism (48, 49). But the usual occurrence of hirsutism (n=4), acne (n=1), and/or obesity (n=3, 1 of whom was hirsute) were not emphasized. Awareness of the Stein-Leventhal syndrome was probably stimulated greatly by their claim of uniform restoration of menses following wedge resection. The terminology for the syndrome gradually changed in favor of “PCOS” in the late 1970’s (49). Though Stein and Leventhal initially suspected an unspecified “hormonal” cause for their syndrome, they did not consider the available hormonal data to be convincing for over two decades (50). In 1958 Janet McArthur and colleagues reported that urinary interstitial cell-stimulating hormone (LH) measured by a prostate and testicular weight bioassay was elevated in the four Stein-Leventhal patients studied (51).

Estrogen-progestin combination oral contraceptive pills are so integral to the medical management of PCOS because of their ability to correct the hyperandrogenism and menstrual irregularity and the story of their development

was so sociologically important that it seems appropriate to review it here. Their development began in the mid-1950s when Syntex and Searle lost the race to commercially produce cortisol to Upjohn (52). Syntex continued to provide Upjohn with substrate progesterone that they synthesized from diosgenin extracted from Mexican *Dioscorea* yams (5), so progestin research was a natural direction. Syntex's Carl Djerassi built on an earlier discovery that removing progesterone's C19 carbon increased its potency and enhanced oral efficacy and synthesized the progestin norethindrone (alternatively termed norethisterone), the first of the "19-nor" progestins to be patented (1951). Searle's Charles Colton then embarked on a systemic program to create a series of 19-nor progestins, culminating in a 1953 patent for the related drug, norethynodrel. This they supplied to Gregory Pincus along with research funding (**Fig. 5**).

Gregory Pincus, PhD (1903-1967), who is remembered as "the Father of the Pill", was "a scientist-statesman of the world who engaged productively in the major endocrinology issues of his time" (53). After their perfused bovine adrenal gland system proved impractical for Searle's commercial production of cortisol, Oscar Hechter and he used the system to elucidate the steps in the biogenesis of corticosteroids and the site of action of ACTH (52, 54).

However, Pincus' major interest was in reproductive endocrinology. He had embarked on studies of fertility in the 1930s that included diverse studies of parthenogenesis (55), the estrogenic properties of phenanthrenes (56) and the sterility of rabbits produced by very high doses of estrogen (57). These studies caught the attention of Margaret Sanger (1879-1966), a pioneer of the women's

rights movement and founder (1920) of the American Birth Control League, the forerunner of the Planned Parenthood Foundation (1942). In 1951 she arranged with the Medical Director of the Foundation to meet Pincus to impress him with the urgent need for an effective means of contraception (52). A hormonal pill was advocated as the ideal contraceptive: harmless, reliable, easy, aesthetic, and separate from coitus. Pincus readily agreed. The Foundation provided the seed money, but the major financial supporter quickly became Sanger's friend and patron, the heiress Katherine Dexter McCormick (1865-1967). She was a prominent early women's suffragette who held a degree in biology from Massachusetts Institute of Technology and provided close scientific monitoring of her grants.

Pincus assembled a contraceptive research team that included his collaborator Min-Chueh Chang, PhD to head screening for potent antifertility agents, the distinguished infertility clinician-investigator Dr John Rock, to direct human contraceptive studies, and Dr Celso-Ramon Garcia to supervise the clinical trials. In 1956 they reported that the 19-nor progestins norethindrone, norethynodrel, and norethandrolone were orally active, potent ovulation inhibitors in animals and in women (**Fig. 5**) (58, 59). When the 1-2% of estrogen recognized to be contaminating norethynodrel was removed, higher rates of bleeding were encountered (52). So Pincus reintroduced mestranol (the 3-methyl ester of ethinyl estradiol) 0.15 mg with 9.85 mg of norethyndrel to form Searle's Enovid (**Fig. 5**) (60), thus producing the first of the estrogen-progestin combination oral contraceptives that are the predominant contraceptives today. As their clinical trials progressed, they reduced the dosage of norethynodrel by three-quarters and mestranol by one-third to counter side effects while maintaining efficacy. In 1957

these three drugs—norethynodrel-mestranol (Enovid, Searle), norethindrone (Norlutin, Syntex), and norethandrolone (Nilevar, Searle)—were shown to inhibit ovulation and cause endometrial hypoplasia (61) and were approved for the treatment of menstrual disorders in women (52).

For contraceptive trials, Pincus chose Enovid mainly because of its high potency and its lack of the mild androgenic effects of the high doses of norethindrone in use at the time, but also partly because of his relationship with Searle (52). In 1956, after a series of false starts due to cultural, religious, and legal barriers, Pincus' team began quickly recruiting for contraceptive clinical trials in Puerto Rico, where well-established birth control clinics were legally run primarily under the auspices of the island's Family Planning Association, and later in Haiti. In 1957 an independent Enovid contraceptive clinical trial was begun in a Puerto Rican local family planning clinic run by a Quaker missionary hospital physician Adeline Satterthwaite and Dr Charles Gamble, an early leader of U.S. birth control movement (62). In 1959 Pincus reported the data on 830 women on Enovid (63, 64). Enovid was approved by the Food and Drug Administration for contraceptive use in 1960. Syntex licensed norethindrone to Ortho Research foundation whose formulation Ortho-Novum was approved in 1962. The final hurdle to widespread adoption of these drugs was overcome when the U.S. Supreme Court, in *Girswald vs Connecticut* (1965), overturned the Connecticut "Comstock law" that had prohibited medical means of contraception as unconstitutional on the grounds of privacy.

3 Early mechanistic research on hyperandrogenic disorders:1965-1972

3.1 Developing assays for androgens in blood

343 Endocrine research in 1965 was very different from what it is today. The National
344 Institute of Child Health and Human Development, which was to become the major
345 source of extramural funding for both general pediatric and reproductive
346 endocrinology research, had only been established 3 years earlier by President John
347 F Kennedy. The discovery of steroid hormone receptors and dihydrotestosterone as
348 the major target cell mediator of testosterone action were 1-3 years in the future
349 (65-67). Androgens were measured for clinical purposes as 17-KS in 24-hr urine
350 collections by a colorimetric reaction (9, 68), and steroid laboratories smelled of
351 urine and such organic solvents as benzene used in column, paper, or thin-layer
352 chromatography. Rosalyn Yalow, PhD and Soloman A Berson, MD had only recently
353 described the radioimmunoassay for plasma insulin (69), for which Yalow would
354 share the 1977 Nobel Prize in Physiology and Medicine. Otherwise, hormones were
355 measured only by labor-intensive methods: whole-animal bioassays for peptide
356 hormones and gas-liquid chromatography and double isotope derivative dilution for
357 specific steroids (70), with the latter showing promise for measuring testosterone in
358 the plasma of women (71). Although the methodology for raising antisera to steroid
359 hormones had been described, radioimmunoassays for them in biological specimens
360 were not yet available (72). The internet did not exist, so literature searches meant
361 methodically paging through the library's *Index Medicus*, so relevant publications
362 were easily overlooked. Manuscripts were prepared on manual typewriters and
363 corrected with white-out and literal cutting-and-pasting; Xerox® copiers were not
364 yet generally available. The Stein-Leventhal syndrome was considered to be in the
365 purview of obstetrics and gynecology specialists, and there was no clear
366 understanding of the nature of its likely endocrine cause (73). Only 66 publications
367 on PCOS were cited by PubMed in 1965 (**Fig. 6**).

368

369 1965 was the year I began training in Pediatric Endocrinology at The Children's
370 Hospital of Philadelphia (CHOP) in the program of Alfred Bongiovanni, MD and
371 Walter Eberlein, MD. That I would embark on a clinical investigational career was
372 unlikely. I had entered medical school as a reluctantly dutiful son of Jewish parents
373 who expected no less from their scholastic bookworm of an oldest child. My father
374 was a Ukrainian immigrant, my mother was American-born of Polish immigrants;
375 they had had established a small-town retail clothing business in central Illinois.
376 None of us knew anything about Medicine. For them it was a matter of family
377 prestige, for me anxiety. I hedged my career options by majoring in English
378 literature (Northwestern University, 1956), thinking I would fall back on teaching it if
379 I were deterred by the challenge of anatomy cadavers. But with my first courses in
380 Pathology, I grew to love clinical medicine and find it endlessly interesting. I spent
381 one summer in a surgical research laboratory where it seemed that no dog survived
382 cardiac surgery. Otherwise, I had no specific research training. I endeavored to
383 master clinical medicine, and after receiving my MD degree (Northwestern, 1960), I
384 entered a rotating internship (Philadelphia General Hospital 1960-61),
385 procrastinating before deciding on specializing in pediatrics. During residency at
386 CHOP (1961-63), I was befriended by the pediatric endocrine fellows, with whom I
387 published my first scientific paper, a case report about two infants with autosomal
388 trisomies, entities only recently discovered (74). I then fulfilled a deferred, 2-year
389 military draft obligation as a US Army pediatrician/general medical officer. I
390 returned to CHOP for pediatric endocrine training. My initial goal was to prepare
391 myself to develop a teaching clinic, and I viewed the 2 years of research training in
392 the 3-year CHOP program as a means of best understanding the field. However, it

393 enabled me to pursue a career as a perpetual student with the intellectual tools to
394 find answers to clinical questions for which textbooks did not provide a satisfactory
395 answer, i.e., a career in clinical investigation.

396

397 Bongiovanni and Eberlein had received the 1957 E Mead Johnson Award of the
398 Society for Pediatric Research for their seminal research in elucidating the causes of
399 virilizing CAH, work begun while in Lawson Wilkins' laboratory at Johns Hopkins
400 University (5, 75). Allen Root, MD had just joined their faculty and was establishing
401 a growth hormone radioimmunoassay, which introduced radioiodine to the
402 laboratory. Bongiovanni would try one new project after another and quickly
403 abandon those that did not pan out, and Eberlein would persevere on a project to
404 the point of regret, so they were very productive as a team; and Root was
405 meticulous. I tried to channel the best traits of these men throughout my career.

406

407 Dr. Jeremy Winter, one year ahead of me in pediatric endocrine training, had been
408 advised by the preceding fellows to pursue the steroid research in which the chiefs
409 excelled. This work was intriguing because I had been promptly introduced to the
410 diagnostic difficulty in clinically distinguishing benign premature pubarche from
411 virilizing CAH, which at the time required sending these children home for 24-hr
412 urine collections to measure 17-KS and pregnanetriol (a specific metabolite of 17-
413 hydroxyprogesterone) to begin ruling out virilizing disorders. I embarked in late
414 1965 on my initial laboratory research project under the guidance of Walter Eberlein
415 and his research associate, the steroid chemist Anne Patti, to develop the
416 preparatory chemical and chromatographic methods for Eberlein to assay children's
417 plasma 17-KS by gas-liquid chromatography. Dr Claude Migeon, then in the Wilkins

418 laboratory (76, 77), had identified them as DHEA sulfate (DHEAS) and androsterone
419 sulfate and quantitated them in adult plasma in 1955-56, using sulfuric acid
420 hydrolysis, paper chromatography and colorimetric methods (78, 79). In 1965,
421 Baulieu reported the results of a series of studies that demonstrated that DHEAS,
422 unexpectedly, was not only a DHEA metabolite, it was secreted by the adrenal
423 gland (80). Our data documenting the rise in plasma DHEAS and androsterone
424 sulfate from childhood during pubertal maturation appeared in 1969 (81).

425

426 When my plasma 17-KS project was well along in late Fall of 1966, Dr, Bongiovanni
427 asked me to set up the urinary aldosterone assay of Dr Ralph E Peterson (82), a
428 double isotope derivative dilution method that had frustrated Bongiovanni's long-
429 time research assistant. , I agreed to establish the aldosterone assay, with the
430 understanding that I would then turn the experience gained towards assaying
431 plasma testosterone. By then the literature showed that most androgenic steroid
432 metabolites in urine were not unique products of secreted steroids (70, 83), so I was
433 convinced that we should be measuring the most potent known androgen,
434 testosterone, in its secreted form in blood, i.e., plasma testosterone rather than
435 urinary testosterone glucuronide.

436

437 By the Spring of 1967, I had established the aldosterone method and was ready to
438 begin my testosterone project. In 1965 Dr Richard Horton, J Shinsako, Peter H
439 Forsham (84) reported that plasma testosterone in normal women could be reliably
440 quantitated using double isotope derivative dilution methodology. This had been
441 closely followed by similar assays and extension to testosterone precursors from
442 several other laboratories (84-90). These investigators performed elaborate

443 determinations of metabolic clearance rates and precursor-product interconversion
444 rates that indicated androstenedione to be the predominant androgenic steroid
445 secreted by the ovary and approximately half of women's plasma testosterone to
446 arise from androstenedione in the peripheral circulation (91). This peripheral
447 conversion primarily occurred outside the splanchnic system in such sites as skin
448 and lungs. In 1966 Horton reported with a reliable method for the first time that a
449 small amount of testosterone was secreted by normal ovaries (92).

450

451 These double isotope methods required 25 mL of plasma in women, far too
452 insensitive to be used for pediatric investigations. This was about to change. Dr.
453 Winter returned from the Spring 1967 FASEB meeting with the news that Horton
454 had reported that testosterone could be measured quickly and directly in 4 mL male
455 plasma using the newly available competitive protein binding (CPB) technique, and
456 Horton soon published this (93). (CPB was a forerunner of radioimmunoassay that
457 used pregnancy plasma as the source of the recently described testosterone-
458 estradiol (sex hormone) binding globulin (SHBG) instead of a specific antibody (91).)
459 Horton's method grossly overestimated the lower plasma testosterone
460 concentrations of women. Upon learning this, I immediately realized that women's
461 samples would require preliminary preparatory chromatography—my recently
462 acquired skill--because other circulating steroids ("17 β -hydroxysteroids") were
463 competing with testosterone for SHBG binding sites.

464

465 By early 1968 I had succeeded in developing a highly specific plasma testosterone
466 CPB method, far more sensitive and rapid than any published testosterone assay. I
467 was hooked on a research career of discovery. My manuscript was submitted in

468 September 1968, by which time I had just begun at the University of Chicago where
469 I was scrambling to establish my new laboratory and Pediatric Endocrinology
470 division. However, I was scooped in August 1968 by Darrel Mayes, PhD, working in
471 the laboratory of Charles A Nugent, MD: they published the first CPB assay specific
472 for plasma testosterone, from which I borrowed their method of using a small
473 amount of ^3H -testosterone to correct for procedural losses (94) (Mayes soon after
474 established Endocrine Sciences (later renamed Esoterix) Laboratories, the first
475 commercial steroid assay specialty laboratory). My simpler method, requiring one
476 thin-layer chromatographic preparatory step and separating free from bound
477 testosterone by a rapid charcoal adsorption method, was published 10 months later
478 (95) and was sufficiently sensitive to measure testosterone in 5 mL plasma from
479 individual prepubertal children (81). This was about 5 years before
480 radioimmunoassays for plasma testosterone and related steroids, which were about
481 10-fold more sensitive, were introduced by Dr Guy Abraham (96).

482

483 **3.2 Premature adrenarche: changing adrenal androgenic response to** 484 **ACTH**

485 Albert Dorfman, MD, PhD, Chairman of Pediatrics at the University of , had recruited
486 me beginning in 1968 at 34 years of age to establish a Pediatric Endocrine Section
487 and had provided me with my own research laboratory on the 5th floor of the new
488 Wyler Children's Hospital and a laboratory technician. My research plan was to
489 develop similar assays of high accuracy, specificity, sensitivity, and precision for
490 testosterone precursors in blood for the study of children with hyperandrogenic
491 disorders. Dr Dorfman helped me formulate these ideas into research grants (my
492 first exposure to strict hypothesis-oriented research!).

493

494 For some time, I had more students and residents than patients in my new Pediatric
495 Endocrine Clinic on Wyler's 1st floor! My fledgling clinical practice gave me time to
496 establish these new CPB assays for androstenedione, DHEA, and DHEAS (97, 98)
497 while soon yielding several girls with premature pubarche to settle the Talbot-
498 Wilkins dispute, ie, test the hypothesis that this was usually due to premature onset
499 of the secretion of adrenal androgens (premature adrenarche) rather than end-
500 organ hypersensitivity to the small normal childhood androgen levels. In 1971 we
501 demonstrated that girls with premature development of pubic hair usually had
502 elevation of plasma DHEAS and DHEA, which indicated premature adrenarche (99)
503 and which differed from the androstenedione-predominant responses of young
504 children to protracted ACTH stimulation (100). These data led me to postulate that
505 adrenarche results from a changing pattern of the adrenal biosynthetic response to
506 ACTH. In 1976, Dr Maria New's group confirmed my steroid findings in a larger
507 series of children using newly available rapid radioimmunoassays (101); they also
508 showed that DHEAS was low in panhypopituitary patients (102). Others held to the
509 view that adrenarche resulted from increasing production of an adrenal androgen-
510 stimulating hormone of pituitary origin (5, 103).

511

512 In 1982, having upgraded from CPB to radioimmunoassays, we published evidence
513 directly supporting our concept: DHEA and 17-hydroxypregnenolone responsiveness
514 to ACTH of children with premature adrenarche were intermediate between those of
515 preschool children and adults. The steroidogenic pattern of precursor/product ratios
516 suggested increased 17, 20-lyase efficiency, decreased 3 β -hydroxysteroid

517 dehydrogenase (3 β HSD) efficiency, and increased sulfotransferase efficiency during
518 adrenarche (104).

519

520 D. Lynn Loriaux, MD, PhD, Dr Gordon Cutler, and their NICHD colleagues obtained
521 complementary data at about the same time. DHEA and DHEAS were stimulated by
522 48 hr ACTH infusions in normal adults, though not by 6-hr infusions in 4-6 yr old
523 children, and ACTH deficiency resulted in more profound suppression of these than
524 of cortisol (105). A later study by this group showed an increase of adrenal
525 microsomal 17-hydroxylase and 17, 20-lyase activities across adrenarche (106). In
526 1985 Dr Jeremy Winter's group described decreased adrenal 3 β HSD activity in
527 adrenal microsomes across adrenarche into adulthood (107).

528

529 Meanwhile, in 1973, Dr Georg Dhom demonstrated that focal development of the
530 ZR begins at 5 yr; its development as a continuous zone is increasingly found from
531 6 yr onwards and is complete by 15 yr (108). He associated this with adrenarche and
532 increasing production of DHEA and DHEAS. Melvin M Grumbach, MD called
533 attention to these histologic data with an influential graphic of the parallel rise re
534 age of his data on serum DHEAS levels with Dhom's data on percent of cases with
535 continuous ZR development (103).

536

537 In an elegant and technically challenging series of papers commencing nearly 20
538 years later, William F Rainey, PhD, Takashi Suzuki, and collaborators demonstrated
539 conclusively that adrenarche is associated with a specific pattern of ZR gene
540 expression (109, 110) that explains earlier predictions (111): increased expression
541 of *cytochrome b5* (which encodes an electron transport protein that promotes

17,20-lyase activity of P450c17 (112)), decreased *HSD3B2* expression (3 β HSD2), and increased expression of *sulfotransferase 2A1* ((**Fig. 7**) (1).

Rainey, Richard Auchus, and their University of Michigan group in 2013 used advanced liquid chromatography tandem mass spectrometry methodology to examine the adrenal effluent and its response to ACTH (113). Thus, they discovered that 11 β -hydroxyandrostenedione, and to a much lesser extent, 11 β -hydroxytestosterone and 11-ketoandrostenedione, are secretory products of the adrenal cortex (**Fig. 7**) (114), not peripheral metabolites of cortisol and corticosterone as had been assumed for decades (Hechter's early finding of 11-ketoandrostenedione—"adrenosterone"-- in the bovine adrenal effluent (54) was overlooked to this day!). Furthermore, they demonstrated that 11 β -hydroxytestosterone together with its more potent peripheral metabolite 11-ketotestosterone rival testosterone in biopotency (1, 113): thus, these are the true adrenal androgens. They then showed that the ZR expresses 11 β -hydroxylase type 1 and 17 β -hydroxysteroid dehydrogenase type 5 (17 β HSD5), which converts androstenedione to testosterone, demonstrating that the ZR is the major source of adrenal androgens (110). Meanwhile, Karl-Heinz Storbeck and associates independently discovered that a castration-resistant prostate cancer cell line converts 11-oxyandrostenediones to 11-oxytestosterones and on to 11 β -hydroxydihydrotestosterone and 11-ketodihydrotestosterone, which are androgen receptor agonists with respectively 47% and 96% the potency of dihydrotestosterone (115).

In 2018 11-ketotestosterone was found to be the main circulating androgen in normal and premature adrenarche by the University of Michigan group, exceeding serumtestosterone levels by averages of 2- and 3-fold, respectively (116). Understanding of the developmental basis for adrenarchal ZR development is currently unclear (1). Understanding ZR function is important for understanding the functional adrenal hyperandrogenism of PCOS, discussed below.

3.3 Plasma free androgen elevation in hirsutism

Soon after arriving at the University of Chicago as the sole pediatric endocrinologist, I began attending the well-established Internal Medicine Endocrine Division's Endocrine Grand Rounds. "Endorama", as it was known to generations of University of Chicago trainees, was then held in the foyer of the General Clinical Research Center. Patients undergoing study were presented in person, and virtually every week we saw hirsute, obese women undergoing urine collections for fractionated 17-hydroxycorticoids and 17-ketoseroids to detect possible Cushing's disease or virilization, investigations that usually yielded no satisfactory answer. These Medicine colleagues gladly sent blood samples to my laboratory for the newly available testosterone determination in the hope of getting answers to the mystifying problem of these patients' hirsutism. Serum testosterone proved to be of only slight added value to urine 17KS, not surprisingly (87). Nevertheless, plasma testosterone and related steroid intermediate assays were of sufficient utility clinically that my laboratory was expanded into a branch of the University Hospital Laboratories, affording me familiarity with all androgen-related clinical problem cases in our university medical center.

591 In 1969, Samuel Refetoff, MD was recruited and established a Thyroid Laboratory
592 that assayed thyroxine and a free thyroxine index by CPB methodology. It was soon
593 clear that the serum free thyroxine index was superior diagnostically to the total
594 thyroxine, in keeping with the accruing evidence that the free (unbound) fraction of
595 plasma hormones was the active moiety and that thyroxine binding globulin was a
596 major determinant of the serum free thyroxine concentration.

597

598 Because of the parallel of testosterone plasma binding to that of thyroxine, it
599 seemed likely that a plasma free testosterone index would prove to superior to the
600 plasma total testosterone level in detecting androgen excess in hirsute women. To
601 test this concept, with Refetoff's advice, I proceeded to modify my testosterone CPB
602 assay to measure plasma SHBG binding capacity and indexes of the plasma free
603 testosterone and free 17β -hydroxysteroid concentration. Indeed, the plasma free
604 testosterone index proved to be elevated 50% more often than the total
605 testosterone in hirsute women, in part because their SHBG binding capacity was
606 significantly decreased compared to non-hirsute women; also the free 17β -
607 hydroxysteroid index was often elevated when free testosterone was not (117).
608 These studies provided the first evidence that hirsutism was usually due to
609 hyperandrogenism. (Several years later, George W Moll, Jr, when an MD, PhD
610 student in our laboratory, demonstrated that the percent of testosterone binding to
611 SHBG determined by our rapid charcoal adsorption method correlated highly with
612 percent free testosterone binding determined in whole serum under physiologic
613 conditions (118). This put our free testosterone assay on a firm physical-chemical
614 footing, and these free testosterone concentration results were consistent with
615 other estimates that appeared approximately concurrently.

616

617 At a site visit to referee my NICHD career development award application (granted
618 1972), Claude Migeon asked how we would quantify hirsutism. Ferriman and
619 Gallwey had previously published UK normative data on a consecutive series of
620 women attending a general outpatient clinic; they devised a semi-quantitative
621 scoring method for hirsutism; they considered the forearms and legs to indicate an
622 “indifferent” score, the nine other nine sites a “hormonal” score (119). Dr. Migeon’s
623 question stimulated me to have a cartoon drawn of the Ferriman-Gallwey hormonal
624 scoring system to facilitate clinical usage. When we eventually published the figure,
625 considering their norms applicable to the general American population (120), it was
626 widely adapted and emulated (121).

627

628 One of my lines of investigation was to search for plasma unconjugated 17β -
629 hydroxysteroids other than testosterone. I started by looking for 5-androstenediol,
630 which had been reported to circulate as a sulfate in human plasma by Reijo Vihko,
631 MD, PhD (122). Women’s plasma concentration of unconjugated 5-androstenediol
632 proved to be greater than that of testosterone (123). (However, our subsequent
633 data indicated that measurement of 5-androstenediol, as well as 5- α -
634 dihydrotestosterone, added very little to the evaluation of hirsute women (124,
635 125).) Though I had evaluated the SHBG-binding of 11β -hydroxyandrostenedione
636 (miniscule) (95), I had concluded that most of the apparent 17β -hydroxysteroid
637 concentration was due to low-affinity binding of steroids with low inherent
638 androgenicity, e.g., DHEA. However, my plasma 17β -hydroxysteroid assay
639 undoubtedly included the androgenic 11-oxy-testosterones of adrenal origin that
640 were unknown until 2013, as discussed above.

641

642 My other main line of research was determining the source of hirsute women's
643 androgen excess. Thus, I unknowingly began to study PCOS.

644

645 **4 PCOS research**

646 **4.1 Mainstream PCOS research, 1965-1990**

647 *Gonadotropins.* Human gonadotropin radioimmunoassays were developed by Dr
648 Rees Midgley and colleagues in 1966-67 (126, 127). Midgley took advantage of the
649 recently recognized high cross-reactivity of antibodies to LH and hCG for his LH/hCG
650 assay (a prelude to the recognition of the structural similarities of the
651 gonadotropins, particularly of LH and hCG (128)) and advances in pituitary
652 gonadotropin preparation by Leo E Reichert for his FSH immunoassay. These
653 radioimmunoassays greatly facilitated reproductive endocrinology research.

654

655 The hypothalamic gonadotropin-releasing hormone (GnRH) was identified and
656 synthesized in the early 1970s by the laboratories of Roger Guillemin and Andrew V
657 Schally, for which these men shared the 1977 Nobel Prize in Physiology and
658 Medicine with Yalow (129-131). On the heels of these discoveries, Ernst Knobil and
659 associates demonstrated in rhesus monkeys that pulsatile administration of GnRH
660 was required for normal gonadotropin secretion and that estradiol not only exerted
661 negative feedback effects on gonadotropins but also induced positive feedback on
662 gonadotropin release in women when estradiol exceeded a threshold value over a
663 critical period of time (132, 133). These principles were soon shown to apply to
664 women (134-138). Conversely, constant, prolonged administration of GnRH
665 paradoxically down-regulated gonadotropin release, a phenomenon that Dr William

666 F Crowley, Jr and Loriaux, later exploited to develop the first specific treatment for
667 central precocious puberty (139, 140).

668

669 Meanwhile, Dr. Samuel Yen and colleagues quickly applied the newly available
670 radioimmunoassays to explore Janet McArthur's 1958 observation of elevated
671 urinary bioassayable LH in Stein-Leventhal syndrome (51). Yen's group reported in
672 1970 that mean serum radioimmunoassayable LH was consistently and significantly
673 higher, and FSH significantly lower, in women with PCOS than in eumenorrheic,
674 follicular phase women (141). They postulated that a disturbance in the
675 hypothalamic regulation of gonadotropins was causally related to the ovarian
676 dysfunction.

677

678 As soon as GnRH became available, Yen's group (1976) used it to demonstrate
679 increased LH responsiveness to GnRH in women with PCOS (142). They proposed
680 that the disturbance in gonadotropin regulation resulted from positive feedback by
681 the excessive acyclic estrone production that arose from peripheral conversion of
682 androstenedione in adipose tissue (142, 143), citing the findings of Pentti Siiteri
683 and Dr Paul C MacDonald who demonstrated that peripheral formation of estrone
684 from androstenedione was increased in obese women (144, 145). Yen's postulate
685 became known as "the estrone hypothesis" (**Fig. 8**) (143). This concept profoundly
686 influenced most diagnostic and research thinking into the 1990s and beyond (19,
687 146).

688

689 Elevated LH or LH/FSH ratio was widely adopted as a diagnostic alternative to
690 demonstration of polycystic ovaries for the diagnosis of PCOS, though discrepancies

691 between gonadotropin and polycystic ovary criteria soon began to bedevil the field
692 (147). Research in PCOS was dominated by attempts to understand the differential
693 regulation of the two gonadotropins in response to one releasing hormone and
694 gonadotropin pulse abnormalities, typified by studies by the prominent
695 neuroendocrine groups led by John Marshall, MD, PhD (148) and Crowley (149).

696

697 However, we were skeptical of the estrone hypothesis as an explanation for PCOS
698 pathophysiology (150). Among other reasons, isolated moderately increased
699 androgen levels had been associated with increased LH levels by Dr James Givens
700 and colleagues (151) and Dr Andrea Dunaif while in training with the Crowley group
701 (152). Dr Jeffrey Chang and colleagues and Dr RB Billiar and an international
702 collaborative group had also shown that manipulating serum estrone levels in
703 women and monkeys did not alter serum LH levels (153, 154). Also studies we
704 began with my colleague Dr Anne Lucky demonstrated that LH radioimmunoassays
705 were plagued by non-specificity for bioactive LH due to molecular heterogeneity in
706 circulating LH isoforms (155-157).

707

708 *Ovaries.* The biochemical basis of the two-cell, two-gonadotropin model ;of ovarian
709 estradiol secretion was formulated in the 1970s (**Fig. 9**) (158). Ovarian androgen
710 secretion was first directly demonstrated to require LH by David Armstrong in 1976,
711 using hypophysectomized rats in induced and synchronized proestrus (159).
712 Armstrong then used established cell culture techniques (160) to demonstrate that
713 ovarian androgen arose from theca cells, which responded to LH (161), while
714 granulosa cells secreted estradiol in response to FSH when supplied with
715 testosterone as substrate (162). Dr Ken McNatty and Anastasia Makris in Dr

716 Kenneth Ryan's laboratory reported in 1980 that human theca and granulosa cells
717 from healthy large (≥ 8 mm) follicles only secreted substantial estradiol when
718 recombined in culture and stimulated with LH and FSH (163).

719

720 Meanwhile, LH and FSH receptor binding to the respective theca-interstitial and
721 granulosa cell compartments of antral follicles during the estrus cycle of the rat
722 were first identified by Rees Midgley in 1973 (164) and later confirmed by binding
723 studies (165). Midgley then examined the basis for the increased LH binding of
724 granulosa cells as follicles enlarge and mature: he showed, in collaboration with
725 Anthony Zeleznick and Reichert, that FSH administered in vivo induced LH receptor
726 binding in granulosa cells (166). In 1979 Greg Erickson and colleagues directly
727 demonstrated FSH induction of LH receptors in cultured granulosa cells, the first
728 biochemical step in follicle luteinization (167). Thus, as follicles enlarge before
729 becoming preovulatory, granulosa cells normally become responsive to LH/hCG.

730

731 Histochemical and molecular genetic studies then showed that granulosa cells
732 express too little P450c17 to form androgen, while theca cells express too little
733 P450aromatase to form estradiol (168-170). Dr Walter Miller's laboratory
734 demonstrated that even the luteinized granulosa cells of periovulatory follicles form
735 no androgen in response to LH or hCG although they form progesterone and
736 estradiol (168).

737

738 Desensitization to LH was first noted in ovarian preovulatory follicles by Hans
739 Lindner's group in the early 1970s (171). Dufau and Catt showed that this
740 "homologous" desensitization in testes is characterized by a loss of LH receptors

741 and a simultaneous down-regulation of steroidogenesis, particularly at the level of
742 17,20-lyase activity (172). The phenomenon was soon demonstrated in men (173,
743 174). Homologous desensitization to LH of theca cells was not described until we
744 stumbled across it in 1990 while studying insulin effects (175).

745

746 Estradiol was the first sex hormone implicated in the mechanism by which
747 homologous desensitization down-regulates steroidogenesis: Onoda and Hall
748 demonstrated in purified pig testicular P450c17 that estradiol inhibited its activities
749 (176). Magoffin and Erickson extended these findings to the rat ovary where
750 estrogens were shown to selectively inhibit thecal androgenic responses to LH at
751 the level of 17-hydroxylase and 17,20-lyase activities (177). Estradiol also had a
752 similar effect on the androgenic response to LH in immature or hypophysectomized
753 rats (178). Dr Eli Adashi first showed that testosterone to inhibited its own secretion
754 by Leydig cells in response to hCG stimulation (179). Androgen receptor agonist
755 treatment was then shown to exert this effect at the level of P450c17 (180) and to
756 exert a similar effect on theca-interstitial cells in culture (181).

757

758 Several peptide hormones were meanwhile identified as up-regulators of ovarian
759 androgen secretion. Inhibins, members of the TGF.- β superfamily, had been
760 identified as the gonadal proteins specifically inhibiting FSH and purified by four
761 laboratories in 1985 (182). It was quickly found to be a secretory product of
762 granulosa cells under the primary control of FSH (183) and to augment LH-
763 stimulated androstenedione production by theca cells in culture (184). Insulin and
764 insulin-like growth factor I (IGF-I) were shown in 1988 to also up-regulate theca cell
765 androgen secretion (158), as discussed in the following section. Erickson and also

766 identified prostaglandin E2 as a stimulus to thecal androgen production in 1976
767 (185).

768

769 *Insulin resistance.* A case series of acanthosis nigricans with extreme insulin
770 resistance was reported by Dr Ronald Kahn and associates in the mid-1970s; two of
771 the six cases had PCOS, an association not discussed (186). Dr James Givens, whose
772 report of an earlier similar case with PCOS was cited by Kahn, then investigated the
773 association of plasma insulin and androgen concentrations in obese control and
774 more obese PCOS women, and his group reported a correlation in 1980 (187).

775 Publications concerning PCOS began to rise thereafter (**Fig. 6**). In 1983 Dr Jeffrey
776 Chang, in a reproductive endocrinology-pediatric endocrinology collaboration with
777 Solomon Kaplan, MD, reported that serum insulin, but not glucose, was elevated in
778 response to a glucose load in nonobese women with PCOS: this was the first
779 evidence of insulin resistance independent of obesity (**Fig. 10**) (188). Dr Andrea
780 Dunaif and colleagues definitively demonstrated that the peripheral resistance of
781 glucose metabolism to insulin of PCOS averaged about 1 SD more than expected
782 from obesity status in 1989 (189). This paper's eye-catching title announced the
783 launch of Andrea Dunaif's career as an independent investigator. She was to
784 become one of the most influential PCOS investigators of the era, starting at Mt
785 Sinai School of Medicine and cycling through The University of Pennsylvania and
786 Northwestern University, where she initiated collaborations with the reproductive
787 endocrinologist-molecular biologist Jerome Strauss III, MD, PhD and biostatistician
788 Margaret Urbanek; the cell and molecular physiologist Jan McAllister and the
789 reproductive endocrinologist-geneticist Dr Richard Legro of Pennsylvania State
790 University, all of whose contributions in various combinations figure prominently

791 throughout this narrative. Strauss has a long acquaintance with PCOS: he knew
792 Irving Stein as his namesake grandfather's close friend from Rush Medical College
793 (class of 1912) through careers as Michael Reese Hospital staff physicians (190).
794

795 In 1983, Drs Robert Barbieri and Ken Ryan recognized that the association of insulin
796 resistance and acanthosis nigricans with hyperandrogenism (hyperandrogenemia,
797 hirsutism and/or menstrual abnormalities), which they termed HAIR-AN syndrome,
798 to be relatively common and overlooked (191). Barbieri, et al then reported that
799 insulin alone or with LH consistently stimulated androgen release from polycystic
800 ovary stromal mince incubations from 4 patients with PCOS, but had inconsistent
801 effects in 4 non-hyperandrogenic women; they were the first to postulate. that
802 hyperinsulinemia may be an important contributor to hyperandrogenism (192). In
803 1984, Dr Jeffrey Flier's laboratory demonstrated insulin receptors in PCOS ovarian
804 stroma (193). In 1988, androgen responsiveness to insulin or IGF-I in synergy with
805 hCG (194, 195) or hLH (196), was established by Dr Eli Adashi's group and ours to
806 be a normal property of rodent theca cells in culture. The small responses to IGF-I
807 or insulin alone were not significant. Furthermore, insulin was equipotent with IGF-I,
808 suggesting that the effect was mediated through the thecal insulin receptor. We
809 further demonstrated that IGF-I reversed the homologous desensitization of LH
810 receptor sites by supraphysiologic LH doses (175).

811

812 *Polycystic ovaries.* In 1962 P.E. Hughesdon published a landmark morphological
813 analysis of the ovaries from 17 Stein-Leventhal ovaries in comparison to autopsy
814 controls (197). While the number of primordial stage follicles was normal, there
815 were about double the normal amount of ripening follicles, predominantly 2-4 mm

816 in size. These were found primarily in the outer cortex where primordial and primary
817 follicles arise, but subcortical dislocation of small follicles was more frequent than
818 normal in polycystic ovaries. The increased number of subsequent atretic follicles
819 gave rise to increased stroma, moreso in the medulla than in the cortex. The tunica
820 was heavily collagenized and thickened by 50%. "Usually much over 10" "cysts",
821 i.e., grossly visible follicles, i.e., at least 2 mm diameter, were found in Stein-
822 Leventhal ovaries. Foci of stromal luteinization were seen in about 80% of cases;
823 theca luteinization was occasional. Corpora lutea were noted in 30% of the ovaries,
824 indicative of past ovulation.

825

826 In 1985-86 the ultrasonographer Judith Adams, DMU in Dr Stephen Franks' research
827 group utilized the recently available real-time ultrasonography technique to non-
828 invasively define polycystic ovary morphology (PCOM) as ≥ 10 cysts 2-8 mm
829 diameter associated with an increased amount of stroma (198, 199). Among 158
830 women who considered themselves normal and were not taking oral contraceptives,
831 PCOM was found in 23%. However, three-quarters of this PCOM group had irregular
832 menstrual cycles, suggesting a relationship to PCOS (200). PCOM by ultrasound was
833 soon validated to correspond to anatomic and histologic evidence of polycystic
834 ovaries in women requiring oophorectomy for diverse reasons (201). Later, Franks'
835 group confirmed the PCOS-type abnormality in the ratio of growing to primordial
836 follicles (197) in cortical biopsies from ovaries identified *a priori* by ultrasonography
837 as having PCOM in ovulatory as well as anovulatory women (202)

838

839 Stephen Franks' group documented the entity of "ovulatory PCOS" in their initial
840 paper (198), heralding Franks' career of elucidating the significance of polycystic

841 ovaries and the regulation of folliculogenesis. In a subsequent series of papers,
842 Franks' group further described this entity. Notably, many had hirsutism with
843 regular menstrual periods, but a low rate of ovulation (198) and significantly
844 increased serum testosterone (199).

845

846 In 1986, Dr. Walter Futterweit reported that virilizing testosterone treatment of
847 women for transgender management was associated with polycystic ovaries (203).
848 This finding became important to our re-thinking of the pathophysiology of PCOS
849 because it was the first indication that polycystic ovaries were the result, not the
850 cause, of androgen excess. McNatty, et al showed a few years later that an atretic
851 follicle is an androgenic follicle (204, 205), so the excess of atretic follicles (197)
852 would be expected to increase follicular androgen formation.

853

854 *Familial clustering.* Familial clustering of PCOS in a pattern suggesting autosomal
855 dominant transmission with variable penetrance gradually increasingly emerged
856 after Givens' 1988 report of 3 families of multi-generational PCOS (206, 207).
857 Franks' and Dunaif's groups were the first to systematically begin investigating
858 families of PCOS probands for traits other than PCOS itself: PCOM (208) and serum
859 testosterone (209) fit this pattern in females in whom the possibility of confounding
860 hyperandrogenic states were eliminated. Early studies by British investigators also
861 suggested male-pattern baldness developing prematurely in the 20s-30s to be the
862 male equivalent of PCOM (208, 210, 211).

863

4.2 Elucidating the steroidogenic dysfunction in PCOS, 1972-1995

My studies into the source of androgen in hirsute women began in collaborations with my Medicine and Gynecology endocrinology colleagues Drs Ed Ehrlich and Robert Cleary. The first of these studies in 1972 showed that the elevated plasma free testosterone of amenorrheic hirsute women usually did not suppress normally after dexamethasone administration to suppress ACTH-dependent adrenocortical androgen production, whereas that of eumenorrheic hirsute women did (212). We ignored a small, significant post-hCG increase of urinary pregnanetriol in the amenorrheic group. Our findings suggested an ovarian source for the excess androgen of amenorrheic hirsute women and was the basis for our subsequent use of a dexamethasone androgen-suppression test to identify it. This study also led us to the realization that the serum androgen level of women was not under tight negative feedback regulation.

After Cleary's departure, I began to focus on the hyperandrogenism of oligomenorrheic women with my new gynecologic colleague Dr Moon Kim. An early finding was that hyperandrogenemia occurred without hirsutism in some oligomenorrheic women (213). This was the first indication that hirsutism, acne, and pattern balding are variably expressed pilosebaceous manifestations of androgen excess. The acne aspect of this formulation owes recognition to Dr Anne Lucky. She was my first associate in pediatric endocrinology at the University of Chicago, but left after a few years to become an "endocrine dermatologist". While in dermatology training at Yale she organized a collaboration to study androgens in adult women with moderately severe acne vulgaris. This showed elevated free

888 testosterone in 24% of these women irrespective of the coexistence of hirsutism or
889 menstrual dysfunction (214).

890

891 Two possible explanations have been proposed for this variable response to
892 androgen. First are target cell events that alter androgen action at the androgen
893 receptor level, such as variations in the metabolism of testosterone to
894 dihydrotestosterone (215) or alterations in androgen receptor signaling (216-219).
895 Second are post-receptor biologic factors in the target organ unrelated to androgen,
896 possibly related to those that determine whether the pilosebaceous unit responds to
897 androgen excess with hirsutism or acne or both (220, 221).

898

899 Meanwhile, Moon Kim had taken the lead in demonstrating that the dexamethasone
900 androgen-suppression test findings identified oligomenorrheic women with similar
901 hyperandrogenic ovarian dysfunction irrespective of the presence of laparoscopic
902 biopsy-defined polycystic ovarian histology, except that those with polycystic
903 ovaries had more severe hyperandrogenemia (222). Our diagnostic approach via
904 androgen levels was not widely adopted, however. To a great extent this was
905 because reliable steroid assays would not become widely available commercially
906 until after 2015 (223), and currently there is still not a standard for free
907 testosterone determinations.

908

909 An ovarian source of androgen excess in hyperandrogenic women, often with an
910 associated adrenal source, was indicated by a number of subsequent studies. Guy
911 Abraham and colleagues performed an uncontrolled study that found “elevated”
912 blood 17-hydroxyprogesterone (17OHP) at baseline and post-hCG in 90% of hirsute

women, irrespective of menstrual status; they interpreted this as indicating the ovary to be the main source of 17OHP in hirsute women but, like us regarding post-hCG pregnanetriol, offered no explanation for this finding (224). Abraham's group then suppressed adrenal function by dexamethasone administration in 32 hirsute women, two-thirds of whom had menstrual disorders: their data suggested an ovarian source for androgens in 56%, most in association with an adrenal source, and a sole adrenal source in the remainder (225). Ovarian and adrenal vein catheterization by Dr Marvin Kirschner and associates indicated that the ovaries were the source of androgen excess in most hirsute women (226). In 1983, Jeff Chang and colleagues selectively suppressed gonadotropins with a long-acting GnRH agonist and demonstrated suppression of serum androgens to castrate levels in typical PCOS patients, while DHEA and cortisol levels were spared, strongly indicating an ovarian origin for PCOS androgens (227).

The nature of the steroidogenic defect in PCOS had long been a subject of speculation. A 1961 report of an elevated ratio of androstenedione to estrogens in follicular fluid suggested the possibility of aromatase deficiency as the cause (228). According to the 2-cell, 2-gonadotropin model of ovarian steroidogenesis, it seemed likely that the commonly used hCG test could not be relied upon to pinpoint the site of ovarian steroidogenic defects.

My early efforts to stimulate coordinated steroidogenesis by both ovarian follicular compartments with an infusion of natural GnRH had proven impractical (229). When the potent GnRH agonist analogues were discovered (140), they struck me as the potential solution to this problem. When my grant proposal to NICHD for this

purpose was flatly rejected (one study section comment was, “Everybody knows the cause of PCOS”. This came as a great surprise to me, but shows how pervasive the estrone hypothesis was), I turned to Jessie Goodpasture at Syntex Pharmaceuticals, with whom Lynn Loriaux had put me in touch, to participate in a research trial of their new long-acting GnRH agonist nafarelin for the treatment of children with central precocious puberty (CPP). Dr Goodpasture was able to garner support at Syntex for my investigator-initiated proposal to pilot-test the initial 24-hr of gonadotropin and steroid responses to nafarelin in children with CPP requiring this therapy. The responses of LH and FSH to a subcutaneous injection of GnRH agonist proved sufficiently great and prolonged to stimulate robust estradiol responses (230).

Then Dr Randall Barnes, who had been recruited to the University of Chicago to work with me by Dr James Schreiber, our recently appointed gynecologic endocrinology section head, applied our new GnRH agonist test to patients with PCOS in comparison to healthy controls. Dr David Ehrmann, an Internal Medicine endocrinology colleague, was recruited to our research group to also compare the responses of men to those of women with PCOS. Eight patients with classic PCOS were studied by Dr Barnes, with and/or without concomitant adrenal suppression by dexamethasone: all were hyperandrogenemic with polycystic ovaries and 7/8 had a high LH/FSH ratio. The response to the LH-FSH rise induced by GnRH agonist of patients with classic PCOS was a previously undescribed pattern of sex steroid secretion (**Fig. 11**) (231): serum 17-hydroxypregnenolone responses were increased significantly compared to those of eumenorrheic women, 17OHP levels were above those of controls in 8/8 PCOS patients and androstenedione was above

control values in 6/8, while plasma estradiol and estrone rose to above average levels (231). These findings were not consistent with a steroidogenic block, the only known paradigm for functional hyperandrogenism. Rather, they suggested dysregulation of ovarian androgen formation, particularly evident at the level of 17-hydroxylase and 17,20-lyase. These had recently been shown in man by Peter Hall and Walter Miller to be two activities of cytochrome P450c17, which was encoded by the same gene (*CYP17A1*) in gonads and adrenal glands (5, 232, 233). We proposed that in PCOS “the regulation of cytochrome P-450c17 is abnormal (and)... this enzyme might be “abnormally stimulated by slightly excessive levels of luteinizing hormone or (be) incompletely down-regulated because of an intrinsic defect in thecal-interstitial cells” (231).

Then David Ehrmann took the lead in our group’s evaluation of 17OHP hyperresponsiveness to the GnRH agonist test as a marker for PCOS in 40 adolescent and adult females with otherwise unexplained hyperandrogenemia who presented to our medical center’s medical, gynecologic, and pediatric endocrine clinics with oligo-amenorrhea, hirsutism, or acne (234). Most (58%) of this diverse population of hyperandrogenic patients had this PCOS-type of functional ovarian hyperandrogenism (FOH), irrespective of the presence of LH excess or PCOM. Oligo-amenorrhea was present in 87% of those with FOH, significantly different than in those without FOH (58%). There was 81% concordance between the outcome of the GnRH agonist test and the peak plasma free testosterone response to a dexamethasone androgen-suppression test, additional evidence that this latter test was a valid alternative test for FOH. One or the other of these two tests were abnormal in 72% of this cohort of hyperandrogenic women that included a broad

988 spectrum of clinical presentations. Only about half the women with FOH had
989 elevated serum LH or PCOM.
990
991 Fifty-eight percent of this hyperandrogenic cohort al also had 17-ketosteroid
992 hyperresponsiveness to an ACTH (cosyntropin) test; in about half the cases this was
993 concordant with the typical type of PCOS response to GnRH agonist (234). We
994 termed this “functional adrenal hyperandrogenism (FAH)”. Most of those with FAH
995 had DHEA-predominant responses that were ≥ 3 SD above average for
996 eumenorrheic healthy controls and so met criteria widely considered at the time to
997 indicate nonclassic (partial) 3β HSD deficiency; however, this interpretation was
998 inconsistent with these women’s ovarian 17OHP responses to the GnRH agonist
999 test, which were usually typical of PCOS (235), rarely suggesting 3β HSD deficiency.
1000 (Sonja Pang, MD and collaborators later showed that only DHEA or 17-
1001 hydroxypregnenolone responses >11 SD elevated indicated HSD3B2 mutations
1002 (236)). Indeed, these results led us to reject our previous alternate hypothesis of
1003 exaggerated adrenarche as the cause of the adrenal hyperandrogenism in women
1004 with hirsutism and acne (237). The most parsimonious explanation for our findings
1005 was that FAH was typically due to the same process that causes the FOH of PCOS
1006 (158, 235). Although this conclusion was disputed by some (238), we have
1007 contended that the pattern of adrenal steroid responses differed from that of the
1008 ovary because of the constraints imposed by the differing enzyme expression
1009 pattern, particularly that of 3β HSD2, of the adrenal ZR and the ovarian theca cell.
1010
1011 The results of our ovarian function tests led us to hypothesize in 1989 that FOH was
1012 central to PCOS pathophysiology (150).In other words, the ovarian

hyperandrogenism, whatever the etiology, was postulated to cause the other key features of the syndrome, namely, the anovulation and the polycystic ovaries (**Fig. 12**) (158) .

As increasing data accrued, we concluded that the testosterone overproduction in PCOS required generalized overactivity of thecal steroidogenesis proximal to P450c17, with the disproportionate 17OHP elevation resulting from the 17,20-lyase activity of this enzyme being the rate-limiting step in androgen formation (158, 239, 240). This required a flaw in the normal process of homologous desensitization and the accompanying steroidogenic down-regulation of P450c17 activity that normally limits the androgenic response to LH excess (175, 181). We also noted an apparently abnormally steep dose-response relationship between LH and 17OHP (158), which suggested that factors other than LH excess contribute to the steroidogenic dysregulation. These considerations suggested that dysregulation of cytochrome P450c17 activity (241) was a manifestation of a general dysregulation of the entire steroidogenic cascade that eventuates in androgen secretion (**Fig. 12**) (158).

At that time several factors were already known to alter the androgenic response to LH. We proposed that these modulated androgen production by theca cells and estrogen production by granulosa cells so as to coordinate them and prevent overproduction of either hormone in order to optimize production of healthy oocytes (158, 234). We postulated that dysregulation of thecal P450c17 activities could result from diverse disturbances that disrupt this normal balance: excess LH stimulation, an inherent dysregulation defect, or intra-ovarian (e.g., estrogen,

androgen, inhibin IGF-I) or extra-ovarian (e.g., insulin, IGF-I) disturbances (**Fig. 9**) (**Fig. 12**) (158, 234). The cytokine TNF α was known at this time to affect steroidogenesis (158); its effect on ovarian androgen synthesis proved to be inhibitory (242). The discovery of the stimulatory effects of diverse obesity-related proinflammatory cytokines was in the future.

4.3 Development of specific criteria for PCOS diagnosis

Shortly after our 1989 report of dysregulation of androgen secretion in PCOS, Drs. Andrea Dunaif, Jim Givens (who had begun to show signs of the early-onset Parkinsonism that would curtail his career), Florence Hazeltine, and George Merriam began organizing an NIH-NICHD Conference on PCOS to which basic science and clinical investigators in the field were invited to contribute; it was held in April 1990 and the proceedings published in 1992 (243). Presentations covered the status of PCOS research, including a report on the status of our ongoing evaluation of hyperandrogenic women by GnRH agonist testing (244). Before closing, a participant survey was taken to facilitate the development of research diagnostic criteria for the syndrome. The general agreement of conferees was that definite or probable criteria for PCOS diagnosis should be: 1) hyperandrogenism, clinical (e.g., hirsutism; 48% of respondents) or biochemical (64%), 2) menstrual dysfunction (52%), and 3) exclusion of other known hyperandrogenic disorders (60%) (245). (The “clinical hyperandrogenism” criterion received such broad support because of the poor state of commercial steroid assays (223).) These “NIH criteria”, as they became known, were the first internationally accepted criteria for the diagnosis of PCOS. The adoption of these criteria ended the usage of LH or LH/FSH ratio as diagnostic criteria.

1063

1064 By 2003 European and American reproductive endocrinologists had become
1065 increasingly aware that the clinical expression of PCOS in the infertility population
1066 was broader than defined by the NIH criteria, and they organized a workshop in
1067 Rotterdam, The Netherlands to address this. They concluded that PCOM was an
1068 important alternative manifestation of PCOS (246). The “Rotterdam criteria”
1069 broadened the PCOS diagnostic criteria to include individuals who had 2 of 3 of the
1070 following features: otherwise unexplained 1) clinical and/or biochemical signs of
1071 hyperandrogenism, 2) oligo- or anovulation, 3) PCOM. This yielded four PCOS
1072 phenotypes, A-D, ranging from phenotype A (the full-blown Stein-Leventhal
1073 syndrome with PCOM) to phenotype D (the non-hyperandrogenic phenotype) (**Table**
1074 **3**). The Rotterdam workshop also recognized that these diagnostic criteria do not
1075 encompass the entire clinical and endocrinological spectrum of PCOS.

1076

1077 The severity of hyperandrogenism is much alike in phenotypes A and B and then
1078 decreases across these successive phenotypes, as does, in most populations, the
1079 severity of insulin resistance, obesity, and LH elevation (247); and diagnostic
1080 specificity of the milder phenotypes is successively less (247). The Androgen
1081 Excess-PCOS Society initially argued against the inclusion of the non-
1082 hyperandrogenic phenotype (248). However, the genetic architecture of the four
1083 phenotypes has proved to be similar (249).

1084

1085 Although it has become apparent that normal ovarian volume falls from mid-
1086 puberty through early adulthood until menopause (250, 251)) and that normal
1087 antral follicle counts are greater with current generation, high-resolution ultrasound

equipment per vagina or magnetic resonance imaging (252-254). Only recently has there been consensus that the Rotterdam criteria be updated to define PCOM in adults on the basis of at least a single ovary with follicle number ≥ 20 with current technology, or, if technically unfeasible, follicle number per (maximal) ovary section ≥ 10 or ovary volume ≥ 10 ml (**Fig. 13**) (255).

Because adult diagnostic criteria for PCOS began to be inappropriately applied to adolescents, I petitioned The Pediatric Endocrine Society to sponsor an international workgroup of stakeholder organizations in adolescent medicine to develop consensus on specific criteria for the diagnosis of PCOS during adolescence. Peter Lee, MD, PhD, Secretary of the PES Board of Directors, shepherded this project, and Selma Witchel, MD became the lead author of the 2015 publication (251). The resultant diagnostic criteria are essentially NIH criteria modified to require persistent evidence of otherwise unexplained hyperandrogenic anovulation, according to age- and stage-appropriate standards. Helena Teede, MBBS, PhD led a later PCOS network in developing international guidelines that included updated criteria for assessing adolescent menstrual criteria (256). Other minor modifications and recommendations for diagnostic work-up and therapy were made by this and other international groups (257). There remains no consensus on criteria to define PCOM in adolescence, although it is clear that pubertal ovaries are on average larger and have higher antral follicle counts than those of adults (250, 258).

4.4 Convergence and elaboration: mainstream PCOS research, ca. 1990-2015

Ovarian function in women with PCOS or polycystic ovaries. During the 1990s, other centers verified and extended our ovarian function findings in women with PCOS. Notably, Lourdes Ibañez, MD, PhD collaborated with Dr Janet Hall and colleagues to report similarly elevated 17OHP responses to leuprolide acetate and hCG in PCOS in comparison to controls (259). Their data provided direct evidence of ovarian androgenic hyper-responsiveness to stimulation by LH. While hCG stimulated estradiol secretion in the early follicular phase of their eumenorrheic controls (259), as we also found (260), we later conducted a small study using a half-maximal hCG test dose and found that it did not stimulate estradiol secretion in controls, only in those with functionally typical PCOS (261); this is consistent with the 2-cell, 2-gonadotropin model, with premature luteinization of follicles in PCOS, *as discussed below*).

To directly examine androgen production by polycystic ovaries from both anovulatory (PCOS) and ovulatory women, Stephen Franks' group examined the steroid output of theca cells during 48 hr of culture from the ovaries of women requiring surgery for nonovarian gynecologic disease (262, 263). Franks' research group was attached to a gynecologic surgical unit and was unique in having abundant access to ovaries classified by polycystic ovary histologic status in addition to ultrasonographic PCOM status. Theca cells from small follicles of polycystic ovaries--independent of ovulatory status--produced significantly more progesterone, 17OHP, and, especially, androstenedione than theca cells from

1134 histologically normal ovaries at baseline and in response to LH stimulation. DHEA
1135 and estradiol production did not differ significantly..

1136

1137 Franks group then tested the hypothesis of an intrinsic abnormality of ovarian
1138 androgen production in women with PCOS and PCOM by performing hCG tests
1139 before and after administration of long-acting GnRH agonist for 1 mo to suppress
1140 endogenous gonadotropin levels (264). Compared to controls, their PCOS and PCOM
1141 groups manifested significant 17OHP hyper-responsiveness to hCG both before and
1142 after GnRH agonist; only the PCOS group also displayed significant androstenedione
1143 hyper-responses both before and after. These studies suggested that polycystic
1144 ovaries have an inherent theca cell defect in steroidogenesis that is more severe in
1145 PCOS. We were concerned that their gonadotropin suppression was too short-term
1146 to “rest” the ovary from long-term gonadotropin excess. Therefore, we performed a
1147 modification of their protocol (261), lengthening the period of gonadotropin
1148 suppression to 3 mo and reducing the hCG test dose to half-maximal. Our data
1149 indicated that the steroidogenic dysregulation pattern of typical PCOS is an
1150 inherent defect.

1151

1152 Judith Adams was recruited from London to MGH in the early 2000s by Drs Janet Hall
1153 and Bill Crowley for a thorough and definitive study of the biochemical features of
1154 PCOM in normal women. They studied former control women who had been found to
1155 have well-defined, regular normal ovulatory cycles and no clinical evidence of
1156 hyperandrogenism in order to compare those with and without PCOM (265). In
1157 2004, they reported that the ovulatory PCOM group had a normal gonadotropin
1158 secretory pattern, but significantly increased baseline total and free testosterone

1159 and DHEAS levels as well as 17OHP and testosterone responses to hCG; they also
1160 had significant evidence of insulin resistance. Dr Roger Lobo and collaborators
1161 reported that ovulatory women with PCOM also had increased LH responses to
1162 GnRH (266).

1163

1164 The MGH group subsequently reported a follow-up of 40 such normal volunteers
1165 after an average of 8 years when they averaged 39 years old to determine whether
1166 PCOM predicted PCOS (267). Eighty percent of these women had experienced
1167 spontaneous pregnancy. Volunteers with PCOM still had significantly higher serum
1168 testosterone, but the prevalence of PCOM had fallen by half and none had PCOS.
1169 Thus, PCOM in women with ovulatory cycles does not ordinarily predispose to PCOS.

1170

1171 In the early 2000s we began phenotyping adolescent and adult PCOS with the
1172 hyperandrogenemic oligo-amenorrheic phenotype (A+B) in comparison to
1173 eumenorrheic controls to characterize the relationship among the heterogeneous
1174 clinical variables that constitute PCOS (90% of cycles in eumenorrheic women are
1175 expected to be normal ovulatory cycles (268)). But first we needed to consider re-
1176 defining ovarian function in normal women: the Adams/Franks' data indicated that
1177 some clinically normal, eumenorrheic women have subclinically ovulatory PCOS
1178 (phenotype C). We used a 36-hr protocol to determine relationships among baseline
1179 hormone levels, glucose tolerance with insulin levels, PCOM, and responses to a
1180 rapid dexamethasone-suppression test, a low-dose ACTH test, and a GnRH agonist
1181 test (269). By this time GnRH agonist testing was performed with leuprolide acetate
1182 after the sale of Syntex led to cessation of parenteral nafarelin production (270).

1183

The first of these data were reported in 2009 and the analysis focused on ovarian function of clinically normal volunteers in relation to PCOM (269). Post-menarchal adolescent and adult data were pooled after finding no significant baseline differences in hormone levels or PCOM prevalence. We found that the distribution of 17OHP responses of non-hirsute eumenorrheic volunteers with PCOM (V-PCOM) formed a distinct population intermediate between those of eumenorrheic volunteers with normal ovarian morphology (V-NOM) and PCOS patients. However, V-PCOM were a heterogeneous population: 53% were functionally normal, with 17OHP responses and free testosterone levels like V-NOM; 25% had mildly elevated free testosterone, thus meeting Rotterdam criteria for PCOS phenotype C (one-third of these had 17OHP hyperresponsiveness to GnRHag testing); and the remaining 22% had 17OHP hyper-responsiveness to GnRHag though normal baseline free testosterone levels. Thus, although we had initially considered PCOM to represent a normal variant, our data were consistent with Franks-Adams' data and a more nuanced concept: eumenorrheic women with PCOM fall on a functional spectrum between unequivocal normal and unequivocal PCOS and that amid this spectrum were some with disturbed ovarian function including sporadic anovulation and ovulatory PCOS. At the conclusion of our studies, 31% of our 67 clinically normal, eumenorrheic volunteers had PCOM (258, 271). An updated analysis of this latter group of eumenorrheic V-PCOM showed that 16% had subclinical hyperandrogenemia and these subjects all had FOH by either GnRH agonist test or dexamethasone-suppression test criteria (**Fig. 14**) (271) .

In 2010 Dr Marcelle Cedars' group studied a large group of regularly cycling ovulatory women and reported that nearly a third had PCOM (272). Testosterone

was significantly elevated, adding to the consensus that asymptomatic ovulatory PCOM are a hyperandrogenic group. Their cohort also had elevated blood anti-Müllerian hormone (AMH) levels, which by this time was known to be elevated in PCOS (273) and had been proposed as a surrogate for ultrasonographic antral follicle counts in PCOS (274) (see *Folliculogenesis*, below).

We added AMH determinations to our evaluation of our study population with frozen serum remaining and reported in 2011 that AMH levels were independently related to polycystic ovaries and ovarian hyperandrogenism (275). AMH levels were modestly increased in V-PCOM, but markedly increased in the presence of ovarian hyperandrogenism (i.e., PCOS) with PCOM. This was consistent with the evidence discussed in the *Folliculogenesis* section and extended it. Our collective experience from testing ovarian function in eumenorrheic volunteers with PCOM is summarized in **Fig. 15,A**: 50% had normal ovarian function in comparison to eumenorrheic volunteers with normal ovarian morphology, 10% had isolated elevation of serum AMH; the other 40% had diverse ovarian function abnormalities related to PCOS.

Returning now to our study of the steroidogenic phenotype of hyperandrogenic oligo-anovulatory PCOS. Our study included 99 consecutively consenting adolescent and adult females with hyperandrogenemic anovulation (269). Eleven were unexpectedly unsuitable for analysis because they had nonclassic CAH (n=3) or had been studied during ovulatory cycles (n=8). Sixty-nine percent had the typical FOH (T-FOH) of PCOS, with elevated 17OHP hyper-responsiveness to GnRHag in comparison to volunteers with normal ovarian morphology. These were termed “functionally typical PCOS”.

1234

1235 We then analyzed the nature of the ovarian steroidogenic dysfunction in the third of
1236 adult PCOS (n=44) with “functionally *atypical* PCOS” who lacked the typical type of
1237 FOH (261) (**Fig. 15,B**). Functionally atypical PCOS differed from functionally typical
1238 PCOS in being significantly more obese (mean body mass index 44 vs 33 kg/m²), yet
1239 indexes of insulin sensitivity were similar. Baseline testing showed significantly
1240 lower ovarian volume and lower LH, total testosterone, androstenedione, and SHBG
1241 levels, yet similar free testosterone levels. GnRH agonist testing yielded responses
1242 similar to controls except for low FSH like typical PCOS. Subgroups of 5-8 were then
1243 challenged with half-maximal hCG and FSH doses while on dexamethasone to
1244 suppress adrenal androgens: this “gonadotropin sensitivity test” (GST) provided no
1245 evidence that the steroid excess occurred in response to gonadotropin. Indeed, the
1246 steroid levels of functionally atypical PCOS were relatively insensitive to the GST:
1247 their steroid responses were similar to those of controls except they lacked controls’
1248 significant 17OHP response to hCG and its enhancement by FSH, and the estradiol
1249 response to hCG+FSH was less than controls. On the other hand, unlike controls
1250 they exhibited inhibin-B hyper-responsiveness to hCG, a typical PCOS-like trait,
1251 though less marked, and consistent with an androgen effect.

1252

1253 :We then repeated the GST of PCOS subtypes after long-term gonadotropin
1254 suppression by GnRH agonist treatment. Functionally atypical PCOS differed from
1255 functionally typical PCOS in that the serum testosterone fall was not significant,
1256 although 17OHP, androstenedione, and estradiol fell did (261). They were also hypo-
1257 responsive to the GST. Inhibin-B responsiveness to hCG did not persist after
1258 gonadotropin suppression.

Responses to low-dose ACTH following short-term dexamethasone were then analyzed in detail in larger age-matched cohorts (n=60) of these groups, including adolescents and preserving the original 2:1 ratio of functionally typical to atypical PCOS (276). The baseline free testosterone of this atypical FOH cohort was significantly lower than that of the typical FOH cohort. Low-dose ACTH led to a lower prevalence of DHEA hyper-responses than found using standard higher doses and a narrower spectrum of steroid secretion, with DHEA the sole hyper-responding 17KS. Dexamethasone suppression test criteria indicated that, despite lacking 17OHP hyper-responsiveness, 60% (12/20) of the functionally atypical PCOS had atypical FOH (A-FOH), i.e., serum testosterone did not suppress to a normal level. Functional adrenal hyperandrogenism (FAH) was found in a similar proportion of A-FOH (3/12) as T-FOH (11/40), 3. FAH alone appeared to be the only source of androgen in 3/20 with functionally atypical PCOS. Five of 20 with functionally atypical PCOS had no detectable ovarian or adrenal source for their hyperandrogenism; this idiopathic subgroup had the mildest hyperandrogenemia (total testosterone, LH, and ovarian volume tended to be normal): excess adiposity itself was the only apparent source for androgen excess. The sources of the hyperandrogenism in this entire age-matched PCOS cohort are summarized in **Fig. 15,B**.

Thus, while two-thirds of PCOS have the typical type of FOH, sometimes with FAH, the other third of hyperandrogenemic oligo-anovulatory (phenotype A-B) PCOS have functionally atypical PCOS and demonstrate considerable functional heterogeneity (261, 269, 276). The atypical group is significantly more obese than those with functionally typical PCOS, half morbidly so. However, their indexes of insulin

resistance were similar to the typical group. Notably, their nearly comparable ovarian androgenic function is maintained in the presence of suppressed LH levels. What might be driving the androgen production of this atypical type of FOH? While insulin resistance surely plays a role, it is no greater than that of typical PCOS and would seem insufficient to maintain a nearly comparable degree of hyperandrogenism in the presence of lower LH levels. Cytokine excess, acting in concert with this group's hyperinsulinism, would seem to be the other stimulus: an increasing number of pro-inflammatory cytokines have recently emerged as steroidogenesis stimulators in the context of obesity, as discussed below, and these would seem to be prime candidates to drive the atypical FOH in concert with this group's hyperinsulinism and normal LH levels (**Fig. 9**). In addition, the enlarged adipose tissue mass itself plausibly directly contributes by producing testosterone from circulating precursor androstenedione, as discussed below.

Folliculogenesis. Endocrinologic evidence of premature luteinization of follicles from women with PCOS was obtained by Debbie Willis and Helen Mason in the Stephen Franks group (277). Whereas follicles from normal ovaries do not secrete estradiol or progesterone in response to LH until they reach 9.5-10mm, those from anovulatory PCOS respond at 4 mm. Polycystic ovaries from ovulatory women, which morphologically do not differ from PCOS polycystic ovaries (202), responded normally. Premature luteinization appears to result from insulin (278) and androgen excess (278, 279), enhancing the induction of granulosa cell LH receptors by FSH (167). Premature luteinization seems likely to be the major factor disrupting selection of a dominant follicle and thereby causing anovulation.

1309 Hyperandrogenemia induced in rhesus monkeys was shown to up-regulate FSH
1310 receptors in primary follicles by Carolyn Bondy's group (280). Hyperandrogenemia's
1311 amplification of FSH action would be expected to aggravate premature luteinization.
1312 It may also partially explain the enhanced responsiveness to gonadotropin
1313 stimulation of PCOS women (280).

1314

1315 In the same model system, Bondy's group also showed, that androgen excess
1316 stimulates recruitment of resting primordial follicles into the pool of growing follicles
1317 (281). Thus, hyperandrogenism directly causes the increased number of small
1318 follicles that constitute the polycystic ovary, supporting Futterweit's earlier finding.

1319

1320 A "Müllerian inhibiting substance" was originally hypothesized by Alfred Jost, to
1321 explain his findings in rabbits undergoing early fetal castration, as the testicular
1322 factor distinct from androgen responsible for inhibiting development of the fetal
1323 Müllerian ductal system (282). It was isolated and purified from calf testes and then
1324 biosynthesized 1976-78 by Dr Natalie Josso as anti-Müllerian hormone (AMH) (283).
1325 In 1999 Alexandra Durlinger and colleagues reported it to play an important role in
1326 folliculogenesis by inhibiting primordial follicle recruitment (284). AMH is first
1327 expressed in primary follicles, output per follicle peaks in preantral and small antral
1328 follicles, and it is no longer expressed in follicles >9mm (285). Serum AMH, thus,
1329 indexes the size of the growing pool of follicles (286). Hyperandrogenism stimulates
1330 the recruitment of primordial follicles into the growth phase (281)

1331

1332 In 2003-2004 Dr Didier Dewailly and colleagues proposed that the androgen-
1333 induced increase in small follicle number was responsible the increased serum AMH

in PCOS (287, 288), but whether androgen excess accounts for the increased AMH secretion per cell of PCOS is not established (289). Further studies indicated that AMH elevation contributes to follicle maturation arrest by inhibiting estradiol secretion via FSH-stimulated aromatase expression and by inhibiting P450c17 expression, while estradiol in turn inhibits AMH secretion (289-291). These relations are illustrated in **Fig. 16**. Recently AMH was found to stimulate GnRH pulsatile secretion in mice, possibly via acting on the AMH receptor found in a subset of GnRH neurons (292).

Dunaif, Urbanek, and colleagues, recently reported that heterozygous AMH or AMH receptor variants with dominant negative signaling activity appeared to cause PCOS in 6.7% of their patients (293, 294). Signaling of two of these variants was recently shown to be reduced approximately 90% due to disruption of normal cell processing of AMH (295).

Insulin resistance. In 1993, Franks' group examined the role of insulin resistance in the menstrual irregularity of PCOS. They performed insulin tolerance tests in two groups of PCOS patients with PCOM, one group with oligomenorrhea and a smaller one with regular menstrual cycles (296). Insulin resistance was only found in the oligomenorrheic group. They concluded that insulin resistance is independent of PCOS and that its presence is related to menstrual regularity.

In 1996 Drs. John Nestler and Daniela Jakubowicz reported the results of a placebo-controlled study to determine whether lowering serum insulin by administering metformin affected apparent ovarian P450c17 activity (297). Metformin, but not

1359 placebo, administration to obese women with PCOS significantly lowered baseline
1360 serum free testosterone and serum 17OHP and LH at baseline and in response to
1361 GnRH agonist challenge. They concluded that decreasing serum insulin ameliorates
1362 hyperandrogenism by reducing ovarian P450c17 activity. This demonstration that
1363 the hyperinsulinemia of insulin resistance seemed capable of causing the apparent
1364 dysregulation of P450c17 and that it was ameliorated by metformin was influential
1365 and popularized the use of metformin for the treatment of PCOS. While the
1366 conclusion was sound, David Ehrmann demonstrated that metformin was only
1367 effective to the extent that it brought about weight loss (298), and metformin
1368 efficacy has always been problematic in our hands. Also it was clear to us that
1369 hyperinsulinism was not the sole cause of P450c17 overactivity because insulin
1370 resistance in relation to obesity status was present in only about half of women with
1371 PCOS (189, 299, 300).

1372
1373 Andrea Dunaif in the early 1990s assembled a group that began addressing the
1374 paradox of hyperinsulinemia amplifying androgen excess in the presence of
1375 resistance to insulin stimulation of glucose uptake in skeletal muscle and fat of
1376 PCOS women (301). In PCOS they found, in relation to age- and weight-matched
1377 controls, a distinctive abnormality of decreased responsiveness to insulin of in vivo
1378 glucose uptake, indexing primarily skeletal muscle insulin action, while PCOS'
1379 insensitivity to the insulin suppression of hepatic glucose production was shared
1380 with obese controls. Subsequently, Bock and Dunaif reported that cultured skin
1381 fibroblasts from PCOS women are intrinsically resistant to the metabolic, but not the
1382 mitogenic, effects of insulin (302).

The molecular mechanisms for PCOS' preservation of mitogenic signaling in the presence of intrinsic resistance to the metabolic effects of insulin was then addressed. In 2002 by Dunaif's group reported, using fibroblasts from PCOS women, that insulin resistance usually results from serine-kinase phosphorylation of the insulin receptor and insulin receptor substrate-1 (303, 304). Walter Miller noted that serine phosphorylation, in contrast to down-regulating Insulin receptor signaling, up-regulated the 17,20-lyase activity of P450c17 and proposed that this might explain the association of insulin resistance with PCOS (305). As attractive as was this hypothesis, their subsequent enzymatic and molecular genetic studies led them to conclude that the main kinase that enhances the 17,20-lyase activity of P450c17 is P38alpha (mitogen-activated protein kinase 14) rather than those kinases implicated in the insulin resistance of PCOS (112, 306). On the other hand, skeletal muscle myotubules have a pattern of insulin resistance that is not attributable to specific signaling pathways according to a study by Theodore Ciaraldi and associates (307).

The question of whether insulin directly acts through its own receptor was addressed by Nestler in 1998. Using highly specific antibodies to the insulin and IGF-1 receptor, his group concluded that insulin acted via its specific receptor (308). However, the physiologic relevance of their observations was suspect because very high insulin doses ($>2 \mu\text{g/ml}$) were required. It was 2014 before convincing direct evidence was developed that insulin acts through its own receptor to stimulate ovarian steroidogenesis: Sheng Wu, PhD and Sara Divall, MD in the laboratories of my former associates Andrew Wolfe, Drs Sally Radovick, and Fred Wondisford used insulin-receptor knockout mice to demonstrate that obesity-induced

1409 hyperinsulinemic hyperandrogenic anovulation is mediated by the theca cell insulin
1410 receptor (309).

1411

1412 *Adipose tissue.* The insulin resistance of adipose tissue is attributable to androgens,
1413 rather than being intrinsic like that of skeletal muscle and liver. In 2007, Dr Anne
1414 Corbould and Dunaif demonstrated that PCOS subcutaneous preadipocytes in
1415 culture had no intrinsic defect in insulin action (310). Corbould then reported that
1416 after differentiating these preadipocytes in culture, androgen treatment blunted
1417 their glucose uptake and maximal response to insulin (311). The mechanism was
1418 mediated by insulin-stimulated phosphorylation of protein kinase C. Meanwhile, Dr.
1419 Peter Arner and colleagues showed that androgens stimulate lipolysis, thus
1420 antagonizing a fundamental insulin action (312).

1421

1422 Bruce Spiegelman's group demonstrated in mice (1993) that obesity is a chronic,
1423 low-grade inflammatory state in which adipose tissue secretes tumor necrosis
1424 factor-alpha (TNFalpha), and that this causes insulin resistance (313). In 2003 it
1425 became clear that this and other inflammatory cytokines like interleukin (IL)-6
1426 originate in macrophages that infiltrate the adipose tissue of obese individuals (314)
1427 (315) and form pro-inflammatory crown-like structures (**Fig. 17**) (316). This process
1428 is exaggerated independently of global obesity in PCOS (316). Serum IL-6 levels
1429 have since been shown to be elevated in PCOS (317).

1430

1431 Dr Frank Gonzalez' studies commencing in 1999 showed that TNFalpha is elevated
1432 in PCOS even in the absence of obesity, which suggests that hyperandrogenism
1433 independently plays a role in provoking chronic inflammation (318, 319). He then

built on then-recent research that indicated that the proinflammatory states of obesity, type 2 diabetes mellitus (T2DM), and PCOS are responsible for an abnormal gut microbiome and gut permeability (320, 321). The latter permits increased serum lipopolysaccharide, while serum IL-22, which is anti-inflammatory, declines (though there is contradictory evidence on this point (317)) due to dysregulated intestinal monocyte function: these changes directly exacerbate both androgen production and insulin resistance. Gonzalez' group then showed that glucose or saturated fat ingestion triggers increased serum levels of lipopolysaccharide and other pro-inflammatory factors, as well as anti-inflammatory factors, often moreso in PCOS than in obesity (320, 322).

Although TNF α inhibits P450c17 activities (242), Dr Antoni Duleba's group demonstrated in rat thecal cells that lipopolysaccharide and interleukin-1 β up-regulate key genes in androgen biosynthesis, including, that encoding the rate-limiting step in cholesterol biosynthesis (*Hmgcr*; hydroxymethylglutaryl-coenzyme A reductase), *Cyp11a1*, *Hsd3b*, and *Cyp17a1* (323). They further showed that the nonsteroidal anti-inflammatory drug ibuprofen, an inhibitor of prostaglandin E2 formation, reversed these effects (324) and significantly reduced serum testosterone in PCOS (325); how the responses to ibuprofen are related to phenotype and obesity status remain to be clarified.

Dr Paul Stewart's group identified adipose tissue as an important site of androgen production in 2004. 17 β HSD5, which forms testosterone from androstenedione, is expressed in subcutaneous fat, where it correlated with an obesity index and increased during adipocyte differentiation (326). Dr Kenan Qin in our group had

1459 identified this enzyme as the major testosterone-forming enzyme of the ovary in
1460 2000 (327) (see next section). In 2009 he and Xiaofei Du then demonstrated that
1461 17 β HSD5 is up-regulated by insulin in both fat and steroidogenic cells (328). Thus,
1462 insulin stimulates fat accumulation by preadipocytes and steroidogenesis via the
1463 same transcription factor, Kruppel-like factor 15, mechanistically linking androgen
1464 secretion and fat

1465

1466 Although the low SHBG in obese individuals was initially attributed to
1467 hyperinsulinemia (329), subsequent evidence suggested that excess glucose and
1468 fructose intake themselves together with cytokines mediate the SHBG reduction in
1469 patients with obesity. David Selva, initially working in Geoffrey Hammond's
1470 laboratory, reported in 2007 that glucose and fructose reduce human SHBG
1471 production by hepatocytes in culture (330). This was mediated by a
1472 monosaccharide-induced increase in lipogenesis that reduced hepatic nuclear
1473 factor-4alpha levels, which in turn attenuated SHBG expression. Selva's group later
1474 showed that the proinflammatory cytokines TNFalpha (331) and interleukin-1 β
1475 promote this process, and adiponectin, an adipose anti-inflammatory cytokine that
1476 counters insulin resistance, has the opposite effect (332). SHBG serum levels in
1477 women also have been shown to have a hereditary component (333).

1478

1479 *Diabetes mellitus*. In the early 1990s we realized that not only was insulin
1480 resistance common in women with PCOS, but T2DM also is common in both patients
1481 and their parents. Two of our Medicine Endocrine fellows, Drs. Niall O'Meara and
1482 John Blackman, were sufficiently impressed with our preliminary presentations to
1483 our joint endocrine conferences that they included some of our women with PCOS in

their ongoing studies of T2DM insulin secretion: they showed that our FOH/PCOS patients had insulin secretory defects characteristic of T2DM (334). From then on Dr Ehrmann took the lead in designing and implementing a series of studies of insulin secretory dynamics in women with FOH. First, he evaluated pancreatic beta cell function during a frequently sampled intravenous glucose tolerance test and showed subnormal insulin release in response to glucose relative to insulin sensitivity in normoglycemic, overweight/obese FOH patients who had a positive family history of T2DM (299). Beta-cell dysfunction in women with PCOS was quickly confirmed by Dunaif and Finegood, who extended the finding to nonobese women with PCOS (335). Next, Dr Ehrmann found that young women with PCOS and T2DM differed from those with PCOS and normal glucose tolerance in having a significant (2.6-fold) higher prevalence of first-degree relatives with T2DM (336). Glucose tolerance was impaired in 45% of 122 young women with PCOS, of whom 10% had T2DM; this was a substantially higher prevalence of abnormal glucose tolerance than expected when compared with age- and weight-matched populations of women without PCOS. After a mean follow-up of 2.4 ± 0.3 years, a subset of these women was found to have a significantly higher 2-hr glucose during oral glucose tolerance testing than during the first test. In a later definitive study of insulin secretory dynamics in women with PCOS and their primary family members, Dr Ehrmann showed that heritability of beta-cell dysfunction is a significant factor in PCOS women's predisposition to type 2 diabetes mellitus (337). These data suggest that T2DM is not intrinsic to PCOS, but occurs at a young age in those with insulin resistance.

Gonadotropin regulation in PCOS. Research in the late 1990s suggested that the increased serum LH of PCOS is the *result* of abnormal sex steroid feedback rather than the *cause* of androgen excess. In 1997 Dr Sarah Berga reported that serum LH level and pulse frequency of PCOS were subnormally sensitive to negative feedback by combined estrogen-progestin administration (338). In a subsequent elegant series of studies, John Marshall's group confirmed these findings and demonstrated that higher concentrations of progesterone are required to suppress LH pulse frequency in the presence of luteal phase estradiol levels in adult women with PCOS than in controls (339). Marshall then took his group further and demonstrated that sensitivity to estrogen-progestin negative feedback was conferred in PCOS by anti-androgen treatment (340). These data indicate that androgen excess interferes with the hypothalamic inhibitory feedback of female hormones. The resistance to estrogen-progestin negative feedback of hyperandrogenemia, while significant, is less consistent in adolescents than in adults (341). This discrepancy between adolescents and adults suggests that resistance to negative feedback is not inherent to PCOS. Rather, it suggests that resistance only becomes apparent as the high sensitivity to sex steroid negative feedback of pubertal maturation develops during puberty.

In the late 1990s, LH levels and pulse amplitude in women with PCOS were found to be negatively related to adiposity (342, 343). Further studies by Janet Hall, MD and colleagues (344) and Dr. Leif Wide and colleagues (345) indicated that this was at least in part due to obesity-related accelerated gonadotropin metabolism (111).

Our current understanding of the pathophysiology of the essential features of PCOS, is based on the above body of knowledge: it is depicted in **Fig. 18**: Any disorder that causes ovarian hyperandrogenism suffices to explain the pilosebaceous and anovulatory manifestations. The hyperinsulinemic insulin resistance found in approximately half the cases aggravates all the clinical and laboratory features of the syndrome: premature luteinization causes the anovulatory symptoms and PCOM frequency to worsen. It appears that two-thirds of the hyperandrogenic oligo-anovulatory forms of PCOS (phenotypes A-B) have functionally typical PCOS indexed by 17OHP hyper-responsiveness to LH, which indicates overactivity of theca cell steroidogenesis through P450c17. Commencing in 1999, the inherent nature of functionally typical PCOS was discovered and much has since been learned about its molecular genetic basis, as discussed below. The remaining one-third of cases have functionally atypical PCOS, the cause of which is less clear. However, the data suggest that obesity is the biggest culprit in most of this latter group: the androgenic dysfunction is milder and is hypothesized to be mediated through insulin resistant hyperinsulinism and pro-inflammatory cytokine excess. 17 β HSD5 in the large adipose tissue depot also excessively forms testosterone from circulating androstenedione, with the hyperinsulinism also promoting this effect.

4.5 Developmental aspects of PCOS

Adolescent PCOS. In reviewing PCOS case histories in 1980, Sam Yen had suggested that the endocrine aberrations of PCOS commonly begin before menarche (143). His patients were often 'overweight' before menarche, their menstrual dysfunction commonly began as a continuation of post-menarchal menstrual irregularity, and hirsutism commonly began at about this time.

1557

1558 The first series of adolescents with PCOS were described by Emans and colleagues
1559 in 1980 using gonadotropin criteria (346) and by us in 1983 using androgenic
1560 criteria (347). Drs Allen Root and Thomas Moshang in 1984 reported 2 teenagers in
1561 whom PCOS developed after central precocious puberty (CPP) and cited two
1562 previous similar case reports (348). However, a 2007 consensus conference of
1563 international experts on CPP found no clear evidence for this association (349). Dr
1564 Dan Apter later teamed with Yen's group to detail adult-like LH dynamics and insulin
1565 resistance in adolescents with clinically typical PCOS (350, 351). Our cumulative
1566 experience with adolescents has been that we have never been able to detect
1567 hyperandrogenism before the peri-menarchal stage of development, but at that
1568 point FOH presents in its fully developed form, indistinguishable from that in adult
1569 PCOS (**Fig. 14**). This view is supported by the Sir-Peterman group's recently
1570 published longitudinal follow-up to adulthood of daughters of women with PCOS,
1571 discussed below (352).

1572

1573 The guidelines for the diagnosis of PCOS during adolescence emphasized
1574 persistence of symptoms as a precaution necessary to differentiate PCOS from
1575 "physiologic adolescent anovulation". This is very appropriate for adolescents with
1576 menstrual disturbances who lack clinical evidence of hyperandrogenism, since
1577 about one-third develop hyperandrogenemia late in prolonged cycles according to
1578 pioneering studies from Drs Stefano Venturoli and Eleonora Porcu (353), and it can
1579 be anticipated that menses in over half of such girls will normalize (354), as does
1580 about 60% of adolescent menstrual disturbance (355). However, although I signed
1581 off on these guidelines, I have always thought the "persistence" criterion is too

widely applied. Some adolescents present during the perimenarchal stage with hirsutism or acanthosis nigricans, with or without a menstrual abnormality, and are found to be hyperandrogenemic. My last original scientific data publication was a follow-up study that included such adolescents in whom we had documented FOH by GnRH agonist test and/or dexamethasone androgen suppression test within two months of presentation (356). At an average of 7.2 years later, all had hyperandrogenic anovulation. This experience indicates that if hyperandrogenemia is accompanied by clinical evidence of hyperandrogenism or severe insulin resistance, it is likely to persist.

Premature adrenarche and PCOS. In 1993 Ibañez and colleagues, following up on their premature pubarche cases after menarche, reported that 45% of them, particularly those with “pronounced” adrenarche, developed hirsutism, oligomenorrhea, and 17OHP hyper-responses to GnRH agonist testing (357). They then launched a series of studies that described the frequent association of premature pubarche and/or adrenarche with hyperinsulinemia (358), reduced fetal growth (359), late development (>3 years post-menarche) of oligo-anovulation (360), and central adiposity (361). They proposed that low birth weight indexed a common fetal origin for these disorders (359, 362) and that when it is followed by early childhood central adiposity it may be linked through insulin resistance to cardiovascular risk, as well as PCOS (359, 363-365).

Subsequent studies in other populations have shown that premature pubarche or premature adrenarche are followed in early adulthood by a high (27-59%) prevalence of hirsutism, significant hyperandrogenemia and insulin resistance, but

1607 not a significantly increased prevalence of oligo-amenorrhea (1, 366-368). Thus,
1608 while these latter studies rule out the A-B hyperandrogenic phenotypes, they have
1609 not definitively ruled out mild adult PCOS C-D phenotypes or determined whether
1610 the source of the hyperandrogenism is adrenal or ovarian, so the possibility of FOH/
1611 PCOS cannot be ruled out.

1612

1613 Yen had proposed as part of his estrone hypothesis that the PCOS began with
1614 exaggerated adrenarche (143). I suspect, rather, that premature adrenarche will
1615 prove in some girls to be the first sign of the dysregulation of steroidogenesis that
1616 later manifests as the FOH of PCOS.

1617

1618 *Studies of PCOS families.* In 2006, our group (369) and later Dunaif's (370)
1619 identified metabolic syndrome (resulting from the combination of obesity and
1620 insulin resistance) as a paternal manifestation. Dysglycemia was more frequent in
1621 fathers than mothers in both PCOS study populations (369, 371). Premature male-
1622 pattern balding was not significant in our study, contrary to earlier reports.
1623 However, severe androgenic alopecia in men appears to be a more accurate marker
1624 (372).

1625

1626 In 2006, Dr. Teresa Sir-Peterman and colleagues began publishing data from a
1627 study of daughters of women with PCOS followed longitudinally in comparison with
1628 daughters of a control group. PCOS daughters had elevated AMH levels at 2-3
1629 months of age and early childhood, suggesting excessive ovarian follicular
1630 development, which is consistent with increased ovarian androgen production
1631 (373). At 6.0 yr mean age, prepubertal PCOS daughters had higher 2-hr post-

1632 glucose insulin levels (374), and at 8.5 yr increased ovarian volume was
1633 documented; these differences persisted into puberty (375). Noteworthy is that no
1634 significant differences in testosterone levels emerged until pubertal stages 4-5,
1635 when 63% and 100%, respectively, of the PCOS daughter groups were post-
1636 menarchal. At that point significantly decreased insulin sensitivity index and SHBG
1637 and increased fasting serum triglycerides, androstenedione, and free androgen
1638 index emerged, as did significantly increased LH and 17OHP responses to GnRH
1639 agonist testing (375). In 2019, when 21 of these PCOS daughters reached
1640 adulthood, 11 had hyperandrogenic oligo-amenorrhea and another 4 met
1641 Rotterdam criteria for nonhyperandrogenic PCOS (**Table 3**) (352).

1642
1643 Monogenic transmission of PCOS is extremely rare. Extreme or atypical features are
1644 suggestive. Deleterious gene mutations causing severe insulin resistance are the
1645 most common risk factors for monogenic PCOS (376). Serum AMH levels are below
1646 average for PCOS in cases with deleterious AMH variants (295).

1647
1648 *Prenatal virilization and PCOS.* Our group noticed that post-menarcheal females with
1649 congenital virilizing disorders often had hyperandrogenic oligo-amenorrhea in spite
1650 of good control of their adrenal hyperandrogenism. Therefore, we tested such
1651 women, most of whom had CAH, for PCOS by performing GnRH agonist tests
1652 coincident with adrenal-suppressive doses of dexamethasone for several days
1653 (377). These women proved to have hyper-responsiveness of LH and 17OHP to
1654 GnRH agonist stimulation. These data suggested that congenital adrenal virilization
1655 programmed the hypothalamic-pituitary axis for hypersecretion of LH and ovarian
1656 hyperandrogenism at puberty (377). Ghizzoni and collaborators subsequently

1657 obtained confirmatory findings in young women with classic virilizing CAH (378).
1658 After presenting our preliminary data at the Endocrine Society 1991 annual meeting
1659 (379), David Abbott was intrigued since he had “inherited” a group of anovulatory,
1660 prenatally androgenized, rhesus monkeys upon joining the faculty at the Wisconsin
1661 Regional Primate Center. We discussed a possible collaboration using GnRH
1662 agonist ; however, this proved to be a poor stimulus to ovarian function in rhesus
1663 monkeys.

1664

1665 Abbott, Dr Daniel Dumesic and colleagues in 2002 reported that hCG testing
1666 demonstrated ovarian hyperandrogenism in prenatally androgenized monkeys
1667 (380). Their further studies in rhesus monkeys showed that prenatal
1668 androgenization from mid-first to mid-second trimester or late-second to mid-third
1669 trimester reproduces the entire reproductive and metabolic spectrum of PCOS,
1670 including adrenal hyperandrogenism, obesity, insulin resistance, defective insulin
1671 secretion, and diabetes mellitus (247, 381-383). As they accrued a large study
1672 population of rhesus females, they documented naturally occurring
1673 hyperandrogenemic oligo-anovulation (i.e., PCOS) in 5% of them, with another 15%
1674 fulfilling Rotterdam criteria, very similar to the proportions of PCOS phenotypes
1675 among affected humans (381). These findings point to PCOS having an ancient
1676 evolutionary origin. However, whether the cause of the spontaneous rhesus PCOS-
1677 like state is DENND1A-related like that in humans remains to be determined.

1678

1679 Prenatal androgenization has now been found to cause PCOS-like dysfunctions not
1680 only in rhesus monkeys, but in every species studied, beginning with sheep by
1681 Vasantha Padmanabhan’s group (384)(385). A novel technique was recently

introduced by Paolo Giacobini's group; they performed prenatal androgenization of mice by inhibiting maternal ovarian and placental aromatase with AMH. This caused PCOS-like features through three generations of offspring (386). Hypomethylation of several genes associated with PCOS was found in these mice. Reversal of this epigenetic imprinting corrected LH, testosterone, and metabolic features, proving that epigenetic mechanisms underlie this model.

The PCOS-like neuroendocrine dysfunction in rats prenatally treated with testosterone was found by Jon Levine's group to be mediated by androgenic suppression of hypothalamic progesterone receptor expression and subsequent LH hypersecretion (387). Using a similar virilization protocol in mice, Rebecca Campbell recently demonstrated that the abnormal reproductive cycling was restored by anti-androgen treatment in adulthood (388). Pam Mellon's group recently knocked out androgen receptor in kisspeptin neurons and showed that virtually all the PCOS-like reproductive features of the prenatal AMH model are mediated through the androgen receptor of hypothalamic kisspeptin cells (389). This seems to explain why targeted deletion of the brain androgen receptor in prenatally dihydrotestosterone-androgenized mice by Kristy Walter's group corrected their reproductive dysfunction (390, 391). Taken together, these studies indicate that continued LH excess is required to maintain the PCOS-like reproductive features induced by prenatal androgenization. Thus, the mechanism for hyperandrogenism in this preclinical PCOS model differs from that of typical PCOS in man, which is due to an inherent defect in theca cells (392) that has genetic determinants, the non-gonadal effects of which remain to be determined, as discussed below.

1707 However, the prenatal administration of androgen in animal models would seem to
1708 directly program for the later development of PCOS-like metabolic disturbances in
1709 these models, which contrasts with the lack of consistent evidence for testosterone
1710 excess affecting metabolism postnatally (393, 394). The window during which this
1711 prenatal programming seems to occur is unusual in rhesus monkeys: throughout
1712 most of mid-pregnancy, unlike the late-first trimester critical period for the classical
1713 induction of genital differentiation by testicular hormones (282).

1714
1715 The extent to which prenatal androgenization models of PCOS are relevant to
1716 human PCOS is currently unclear because there is neither obvious nor consistent
1717 evidence of prenatal androgenization in ordinary human PCOS (247). Furthermore,
1718 maternal transfer of testosterone to the fetus is hindered by the high aromatase
1719 activity of the placenta, and fetal ovarian follicle development does not begin until
1720 mid-gestation, after which the ovary is normally inactive until term (247). Of course,
1721 the possibility exists that endogenous up-regulation of fetal ovarian steroidogenesis
1722 by the aberrant *DENND1A* splicing which underlies androgen excess in typical PCOS,
1723 (395), discussed below, occurs mid-gestation. Another possibility would be that
1724 small molecules, e.g., prostaglandin-E2, that mimic or mediate testosterone action
1725 cross from the maternal to the fetal side of the placenta and act via an epigenetic
1726 mechanism, as discussed below.

1727
1728 *Disturbed fetal nutrition.* Ibanez' proposal that low birth weight is a risk factor for
1729 PCOS growth (359) has been supported in some populations, not in others (247). In
1730 some studies, high birth weight has been associated with PCOM and PCOS (396,

397); it is possible that this is related to gestational diabetes, which is associated with obesity, insulin resistance, and diabetes in offspring (398, 399).

Obesity. Obesity is the major postnatal environmental factor in PCOS (247). Obesity emerged as a potential public health problem in the United States and the United Kingdom in the mid-1970s and as a worldwide problem in 1995 (400); it was characterized as an “obesity epidemic”, a term first cited in PubMed one year later. The rare childhood obesity syndromes of pseudo-Cushing’s syndrome and pseudo-acromegaly that are due to severe insulin resistance herald the development of PCOS at puberty (401). Obesity in older children is a risk factor for obesity (402) and thus for PCOS.

Clinically, most obesity seems to be behavioral in origin. However, obesity is itself a complex trait with heritable as well as environmental contributions (403). Whether the obesity of PCOS and their families (369, 370) is primarily behavioral or hereditary is unknown. Yee-Ming Chan, MD, PhD and associates recently used a novel approach to address this issue (372). They applied genetic risk factors for PCOS in women, as determined in the largest available genome-wide association study of that disorder, calculated individual polygenic risk scores for PCOS, and in the general male population found that increase of these risk scores was highly associated with increased odds for obesity. This paper provides convincing evidence that the familial relationship of paternal obesity to PCOS has important genetic determinants.

1755 Weight-loss and bariatric surgery—like all other treatments that cause a reduction
1756 in serum insulin levels-- whether by administration of somatostatin, metformin, or
1757 insulin-sensitizing thiazolidinediones--significantly improve ovulation and
1758 hyperandrogenemia in PCOS (158, 297, 404-408). However, the weight loss
1759 achieved by medical treatment has been modest, averaging about 5 kg, so only
1760 about half of PCOS patients experience improvement in the PCOS symptoms when
1761 they lose weight, and patients with the least severe ovarian dysfunction are those
1762 most likely to benefit symptomatically from weight loss (409). A new era of
1763 treatment with potent glucagon-like peptide-1 agonists (410) carries the promise of
1764 learning more about the contribution of obesity to PCOS.

1765

1766 *Epigenetic factors in PCOS.* Epigenetic factors have been shown to contribute to
1767 many of the intrauterine and postnatal environmental factors noted above to be
1768 related to PCOS. Giacobini's prenatal androgenization mouse model of PCOS was
1769 reversed by correcting the abnormal methylation of these mice, demonstrating that
1770 epigenetic changes induced by androgen were responsible (386). This study also
1771 showed that that several genes found to be hypomethylated in the mice were also
1772 hypomethylated in women with PCOS. Sir-Peterman's group found that prenatal
1773 dihydrotestosterone-treatment of mice led to transgenerational PCOS-like changes
1774 that were accompanied by transgenerational change in expression of several oocyte
1775 genes that were the same as imprinted genes found in adipose tissue of PCOS
1776 patients and serum of their daughters (352), though different than the imprinted
1777 genes in Giacobini's study.

1778

1779 Prostaglandins have been demonstrated to mediate the epigenetic changes induced
1780 by prenatal androgen in brain in a series of studies by Margaret McCarthy's group of
1781 the mechanism of masculinization of behavior (411, 412). There is also evidence
1782 that prostaglandins may mediate androgen effects on the prostate (413).

1783

1784 Disturbed fetal nutrition also has epigenetic-mediated consequences. Heijmans, et
1785 al demonstrated that periconceptual exposure to famine during the Dutch Hunger
1786 Winter of 1944-45 was associated with hypomethylation of the IGF2 gene (414).
1787 Maternal diabetes is associated with persistent epigenomic signatures in metabolic
1788 and developmental pathways (399)

1789

1790 Epigenomic alterations have additionally been indicated in PCOS granulosa cells by
1791 >100 differentially methylated sites affecting a wide variety of functions (415),
1792 including abnormal methylation of ovarian aromatase, AMH and its receptor, and
1793 genes involved in insulin/IGF signaling (416). Epigenomic alterations have been
1794 suspected as the cause of androgen receptor splice variants (216, 217).

1795

1796 **4.6 From phenotype to the biological, biochemical, and molecular genetic** 1797 **basis of PCOS, 1999-ca. 2015**

1798

1799 With the demonstration that “augmented androgen production is a stable
1800 steroidogenic phenotype of propagated theca cells from polycystic ovaries”, the
1801 biological basis of the PCOS phenotype A was revealed in 1999 by the laboratory of
1802 Jan McAllister in collaboration with Jerome Strauss and Richard Legro (392). The
1803 McAllister laboratory had succeeded in establishing theca cell lines from the follicles

of control and PCOS patients with PCOM that could be stored frozen and studied after passaging 3-4 times in culture. The passaged theca cells from women with PCOS constitutively overexpressed all theca cell steroidogenic enzymes and their mRNAs from cholesterol (P450scc/*CYP11A1*) through androstenedione (P450c17/*CYP17A1*), and progesterone, 17OHP, and testosterone production per cell was markedly increased. Forskolin, a cyclic AMP analogue used as an LH surrogate, stimulated pregnenolone and DHEA metabolism by these cells and augmented their expression of *CYP11A1* and *CYP17A1* more than in normal theca cells. Further studies showed that forskolin-stimulated *CYP17* promoter activity was increased in PCOS theca cells, but no such changes in steroidogenic acute regulatory protein activity were detected (417). This *in vitro* biochemical phenotype would seem to account for the *in vivo* secretory phenotype of typical PCOS. McAllister's findings indicate that the theca cell defect in PCOS is constitutive and, hence, inherent.

In 2000, the gene for the testosterone-forming enzyme 17 β -HSD type 5, structurally aldo-ketoreductase 1C3, encoded by *HSD17B5/AKR1C3*, was identified in a human ovary library by Dr Kenan Qin in our laboratory (327). Subsequently, in collaboration with McAllister and colleagues, we demonstrated it to be localized to the theca cells of the ovary (418). Their concurrent biochemical studies indicated that the primary factor driving increased testosterone production by PCOS theca cells passaged in long-term culture was increased production of precursors by increased 3 β HSD and P450c17 activities, not increased 17 β HSD activity (**Fig. 19**).

This McAllister paper indicated that molecular genetic studies would be necessary to reveal the cause of PCOS. Thereafter, the pace of research into the disorder

began accelerating (**Fig. 6**). Multiple plausible candidate genes were evaluated, but results could usually not be replicated (419). As a consequence of the frustration with this approach, a consensus emerged in the PCOS research community that large scale genome-wide association studies (GWAS) would be required to solve the problem. I was skeptical of the quality of the data going into such databases, particularly about the fuzziness in the inclusion of “clinical hyperandrogenism” in the diagnostic criteria and the inclusion of the non-hyperandrogenic D phenotype; it turned out that my skepticism was unwarranted because of the large size of the databases that were developed.

The first large-scale collaborative GWAS was conducted by Zi-Jiang Chen and Yongyong Shi in Han Chinese populations in 2011-2012 and yielded several previously unsuspected genetic loci (420, 421). The strongest linkage in Han Chinese was replicated in European populations and was associated with an intronic 9q22.32 locus within the *DENND1A* (differentially expressed in normal and neoplastic development, isoform 1A) gene (422, 423).

The *DENND1A* linkage led McAllister and colleagues to the discovery of a previously unknown steroidogenic regulatory pathway. They reported in 2014 that *DENND1A* is normally expressed in passaged theca cells predominantly as the *DENND1A.V1* isoform, but a normally less abundant splice variant, *DENND1A.V2*, is constitutively overexpressed in passaged theca cells from the polycystic ovaries of women with PCOS (395). Critically, they further demonstrated that experimental manipulations of the expression of this V2 isoform account for the biochemical phenotype of these PCOS theca cells. Thus, dysregulated *DENND1A.V2* expression appears to account

1854 for the functionally typical type of PCOS we had defined by GnRH agonist testing 25
1855 years prior. *DENND1A* is a member of the *connecdenn* family of proteins, which are
1856 clathrin-associated, adjacent to the inner cytoplasmic membrane, and involved in
1857 protein trafficking, endocytotic processes, and receptor recycling (424). Thus,
1858 *DENND1A* is positioned to affect LH receptor signaling,

1859

1860 McAllister's laboratory subsequently reported that *DENND1A.V2* is also expressed in
1861 adrenal ZR and human virilizing adrenal carcinoma cells (424, 425). Its forced
1862 expression in transgenic mice drives *CYP17A1* expression and androgen production
1863 in mouse ovaries and adrenals (426). They also demonstrated that *DENND1A.V2*
1864 accumulates in theca cell nuclei after gonadotropin stimulation, suggesting that it
1865 may act directly on gene transcription (427).

1866

1867 Matthew Dapas, Geoffrey Hayes, Margaret Urbanek, Andrea Dunaif and associates
1868 in 2019 analyzed whole-genome screening data for *DENND1A* variants in 261
1869 individuals from 62 families. They found that half these PCOS families had one or
1870 more of 32 different *DENND1A* variants, most of which altered *DENND1A* affinities
1871 for transcription factors or RNA binding proteins (428). They proposed that these
1872 variants plausibly drive *DENND1A.V2* overexpression via posttranscriptional
1873 regulation.

1874

1875 Dapas, Dunaif, et al in 2020 then reported an examination of an international GWAS
1876 database of variously defined PCOS cases to identify the relationship of clinical
1877 subtypes to deleterious *DENND1A* variants (429). Their preliminary analysis showed
1878 that the genetic architecture was similar in Rotterdam phenotypes A-B and

phenotypes C-D or by self-report for 13 of 14 susceptibility loci. A PCOS trait analysis showed that ovulatory dysfunction and PCOM were genetically similar for 7 of 8 gene susceptibility loci. They then performed an unsupervised cluster analysis in a cohort of 73 families in which the women were completely genotyped among the 893 United States and European PCOS cases with phenotypes A-B that had complete data for key traits. This analysis identified a “reproductive” subtype that was characterized by higher LH and SHBG with relatively low BMI and insulin levels than the opposite cluster, the “metabolic” subtype. Between these was an “intermediate” subtype with indeterminate results. *DENND1A* variants were found in 65% of the 17 families with the reproductive subtype, which was significantly more than in the other subtypes: there *DENND1A* variants were found in 27% of 22 families with the metabolic subtype and 35% of the 34 families with the intermediate subtype.

Meanwhile, painstaking research by the McAllister laboratory revealed a network of factors that modulates the expression of *DENND1A*.V2--and, thus, ultimately *CYP17A1* expression and P450c17 activity, some of them directly (430, 431). This *DENND1A* regulatory network includes several proteins and nucleotides that had themselves been significantly linked by GWAS to PCOS: those for the LH receptor (*LHCGR*), the zinc finger transcription factor *ZNF217*, the micro-RNA *miR-130b-3p*, and Ras-related protein *RAB5B*. This network interacts with mitogen-activated protein kinase (MAPK) and extracellular regulated kinase signaling to increase androgen secretion (430, 432) and links via MAPK to the insulin mitogenic signaling pathway (430).

More recently, McAllister and Strauss identified more candidate genes by plumbing their trove of passaged theca cells. With H Alan Harris and others (433) they used whole exome sequencing to identify a chromosome 12q13.2 haplotype containing single-nucleotide variants of the *RAB5B*, *ERBB3* (*erb-b2* receptor tyrosine kinase 3), and *PAG4* (*prostate-associated gene 4*) genes that were significantly associated with androgen production by these cells; *PAG4* was differentially expressed although it had not been previously identified as PCOS-associated. *PAG4*, like *ERBB3*, is a target of ZNF217, and so these studies extend the scope of the DENND1A regulatory network. With Harris, McAllister and Strauss also demonstrated, using RNA sequencing of single theca cells, that over a hundred genes involved in androgen formation, from cholesterol acquisition to enhancement of *CYP17A1* and its 17,20-lyase activity, were differentially expressed in PCOS, and this appeared to be driven by increased levels or activity of the transcription factors SREBF1 (sterol regulatory element binding transcription factor) and GATA6 (GATA binding protein 6) (433). This conclusively demonstrates that dysregulation of P450c17 is the end-point of a generalized dysregulation of theca cell steroidogenesis; notably, the data were compatible with heterogeneity in *DENND1A*-dependence.

5. Conclusions and a look forward to research opportunities

It is now possible to place past research on the PCOS clinical phenotypes in relationship to recent developments in molecular genetic PCOS research. Our studies of ovarian and adrenal androgenic secretory function have shown that two-thirds of women with PCOS phenotypes A-B have a functionally typical FOH/PCOS indexed by 170HP hyper-response to LH that indicates generalized overactivity of

theca cell steroidogenesis (**Fig. 15,B**). The studies of McAllister and colleagues indicate that overexpression of the *DENND1A.V2* splice variant found in patients with phenotype A causes a theca cell steroidogenic phenotype similar to the steroidogenic secretory pattern of the FOH found in PCOS phenotypes A and B (392, 395). The 2020 GWAS database analysis by the Dapas, Dunaif and collaborators suggests that two-thirds of PCOS phenotypes A-B constitute a “reproductive” subtype that is related to expression of relatively common intronic deleterious *DENND1A* gene variants (428). The discovery of a *DENND1A* regulatory network in which factors as diverse as microRNA-130b-3p and ZNF17 transcription factor were differentially expressed in PCOS was just then beginning to emerge (430, 431). These latter molecules jointly repress transcription of the *DENND1A.V2* isoform, Deleterious variants of other genes associated with PCOS have recently been identified (434), so the extent to which PCOS phenotypes A-B are due to adverse variants within the *DENND1A* regulatory network or in other adverse variants is unexplored.

What, then, is the cause of the one-third of PCOS phenotype A-B cases with functionally *atypical* FOH (261, 275, 276) (**Fig. 15,B**), which are on average slightly milder than those due to the functionally typical type? Recent data sheds light on this, too. For one, the functionally atypical PCOS group shares several of the characteristics of the Dapas-Dunaif “metabolic” PCOS subtype that has a significantly lesser relationship to adverse *DENND1A* variants (435): functionally atypical PCOS are more obese and have lower SHBG and less significant LH elevation than functionally typical PCOS. In addition, like the Dapas-Dunaif “intermediate” PCOS subtype that has features which overlap both their

1954 “reproductive” and “metabolic” subtypes, the atypical FOH group has some
1955 features of functionally typical PCOS: significantly increased indexes of insulin
1956 resistance, lower FSH levels, and increased inhibin-B responsiveness to FSH
1957 compared to controls (although a significantly lesser one than the functionally
1958 typical group); a few also had the typical PCOS type of FAH.

1959

1960 Consequently, it is plausible that obesity plays an important *causative* role in the
1961 functionally atypical FOH that is responsible for one-third of PCOS phenotypes A-B.
1962 Obesity can cause ovarian androgen excess via a combination of insulin-resistant
1963 hyperinsulinism amplifying the effect of normal levels of LH and of proinflammatory
1964 cytokine excess stimulating generalized theca cell steroidogenesis. Whether obesity
1965 alone is sufficient to explain the degree of hyperandrogenemia manifest in these
1966 patients remains to be determined.

1967

1968 In view of the fairly common prevalence of adverse *DENND1A* variants, a plausible
1969 hypothesis would be that the severity of PCOS manifestations—along a spectrum
1970 from isolated PCOM to severe PCOS phenotype A--depends on a combination of the
1971 “dosage” (a large dose of weakly active variants or a small dose of potent variants)
1972 of common deleterious *DENND1A* gene variants or rare other gene variants, e.g., in
1973 the *DENND1A* regulatory network or AMH-related, interacting with a spectrum of
1974 excess adiposity (**Fig. 20**). A second reasonable hypothesis would be that obesity
1975 and insulin resistance are common in PCOS because the signaling pathways of
1976 these PCOS-related gene variants intersect with the genetic determinants of obesity
1977 and insulin action, ie, if it were not for these gene variants, the association of PCOS
1978 with excess adiposity and insulin resistance would be simply a matter of chance.

1979

1980 Research will of course be necessary to test the above hypotheses. Many other
1981 questions about the pathophysiology of PCOS remain to be addressed other than
1982 these. For example, what is the explanation for elevated AMH levels in
1983 normoandrogenic women with PCOM? Is this an indicator of ovarian androgen
1984 excess too small to be reflected in peripheral blood and/or an indicator of
1985 independent factors determining the inborn size of the oocyte pool? Are there
1986 specific gene variants that label an individual's PCOS carrier status?

1987

1988 Other important overlooked areas of clinical hyperandrogenism research that
1989 warrant scrutiny have been largely ignored because of endocrinologists'
1990 preoccupation with oligo-anovulatory PCOS. We still are faced with the enigma of
1991 "idiopathic hirsutism". The murky understanding of this problem is indicated by the
1992 differences of opinion about its definition. The term has historically been variously
1993 applied to eumenorrheic hirsute women without a polycystic ovary or those with
1994 documented normal ovulation (436). For the hirsutism task force of the Endocrine
1995 Society, idiopathic hirsutism was defined as "hirsutism without hyperandrogenemia
1996 or other signs or symptoms of an a hyperandrogenic endocrine disorder" (437) ,
1997 which reflects the evidence that it arises either from an alteration in the mechanism
1998 of androgen action or in the post-receptor biological response to androgen within
1999 the hair follicle (215, 221). The invocation of ovulation and PCOM as criteria for
2000 diagnosing whether hirsutism is due to androgen excess tells us about the
2001 limitations of our current diagnostic tools. Similarly, it is archaic that hirsutism is still
2002 used as a surrogate for androgen excess. The application of high-quality, liquid
2003 chromatography-tandem mass spectrometry assays for testosterone and 11-

2004 oxytestosterones (438) along with reproducible methods for measuring their binding
2005 to serum SHBG would be expected to discriminate those whose “idiopathic
2006 hirsutism” is due to elevated levels of historically unmeasured androgens from
2007 those who are truly normoandrogenemic..

2008

2009 A related clinical problem that has been overlooked is that of determining the
2010 source of androgen excess in women with eumenorrheic hyperandrogenic hirsutism
2011 or acne vulgaris. These clinical problems, like idiopathic hirsutism, have typically
2012 been the purview of dermatologists. But the endocrinologic basis for these begs to
2013 be reexamined closely. Most probably have androgen excess of adrenal origin (212)
2014 due to the type of functional adrenal hyperandrogenism that now seems to be
2015 related to PCOS (237), but the FOH typical of PCOS is probably present in about
2016 15% in spite of eumenorrhea (234).

2017

2018 We are also still uncertain about the etiology of premature adrenarche. Knowledge
2019 about the
2020 factors determining the apparent premature maturation of the adrenal ZR remains
2021 as meager as
2022 our understanding of the normal development of this adrenal zone (1), and the
2023 possible relationship to PCOS remains to be elucidated. There are interesting roads
2024 for exploration ahead.

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Table 1. Major Milestones in Premature Adrenarche Pathogenesis Research through 2015		
Publication year	Milestone	Project leader
1888	Description of the adrenocortical zona reticularis	Arnold
1942	Pubic hair onset independent of gonadal function termed “adrenarche” and ascribed to adrenal androgen-like hormones	Albright
1952	Isolated premature pubic hair development attributed to increased 17-KS output and termed “premature adrenarche”	Talbot
1955	DHEAS found to be the major plasma 17-ketosteroid	Migeon
1960-65	DHEAS discovered to be a secreted adrenal steroid	Baulieu
1971	Plasma DHEAS & DHEA found to be disproportionately elevated in premature adrenarche, which differs from the androstenedione predominance in children post-ACTH	Rosenfield
1973	Development of continuous zona reticularis attributed to	Dhom
1981	Increasing DHEA response to ACTH found across	Rosenfield
1981	Increased adrenal microsomal 17-hydroxylase and 17,20-lyase activity found across adrenarche	Loriaux
1985	Decreasing adrenal 3 β -hydroxysteroid dehydrogenase activity described from adrenarche into adulthood	Winter
2000	Discovery of the zona reticularis-specific enzyme expression pattern that underlies adrenarchal steroid	Suzuki, Rainey
2013	Discovery of adrenal 11 β -hydroxyandrogen secretion	Rainey
2013	Discovery of high bioactivity of 11 β -hydroxy- and 11-keto-testosterone and dihydrotestosterone	Rainey, Storbeck

2043

Table 2. Major Milestones in PCOS Pathogenesis Research through 2015		
Publication year	Milestone	Project leader
1935	Stein and Leventhal describe 7 patients with amenorrhea and polycystic ovaries $\pm$ hirsutism or acne $\pm$ obesity	Stein & Leventhal
1958	Elevated urinary LH by bioassay in Stein-Leventhal syndrome	McArthur
1966	Testosterone secretion reported by normal human ovaries	Horton
1970	Elevated serum LH and LH/FSH ratio by radioimmunoassay	Yen
1971	Free testosterone index elevated in most hirsute women	Rosenfield
1976	Association of acanthosis nigricans with extreme insulin resistance; two of six cases had hirsutism and polycystic	Kahn
1980	Blood insulin and androgen levels correlate across obese control and PCOS women	Givens & Kitabshi
1983	Insulin resistance in nonobese PCOS	Chang
1983	Acanthosis nigricans, insulin, hyperandrogenism association reported to be common in PCOS	Barbieri & Ryan
1985	Polycystic ovary morphology (PCOM) is defined by ultrasonography and reported in both anovulatory and	Adams & Franks
1986	Insulin stimulates androgen secretion by PCOS stroma	Barbieri
1986	Polycystic ovaries found in testosterone-treated transsexuals	Futterweit
1989	Dysregulation of ovarian P450c17 described in classic PCOS	Barnes & Rosenfield
1992	First expert conference-generated PCOS diagnostic criteria for research ("NIH criteria")	Dunaif
1994	Congenital virilization reported to cause LH excess and PCOS-like ovarian dysfunction	Rosenfield
1995	Type 2 diabetic secretory defects reported in PCOS who have diabetic primary relatives	Ehrmann
1997	PCOS women found to have neuroendocrine resistance to negative feedback by estrogen-progestin	Berga
1998	Androgens stimulate growth of preantral and small follicles	Bondy

1998	Granulosa cells prematurely luteinize in anovulatory PCOS	Willis, Mason, & Franks
1999, 2001	PCOS theca cells constitutively over-express most steroidogenic enzymes, especially P450scc and P450c17	McAllister
2000	17 β -hydroxysteroid dehydrogenase type 5 (HSD17B5, AKR1C3) found to be the ovarian testosterone-forming	Qin & Rosenfield
2000	Anti-androgen reported to reverse neuroendocrine resistance to negative feedback by estrogen-progestin	Marshall
2003	AMH elevation in PCOS linked to excess small follicle number	Dewailly
2004	17 β -HSD5 expression found to be increased in subcutaneous adipocytes in obesity	Stewart
2004	Rotterdam diagnostic criteria for PCOS by international reproductive endocrinology workshop expanded the phenotype to include PCOM as evidence of the disorder	Fauser
2009	Delineation of a functionally atypical biochemical PCOS phenotype	Rosenfield
2011, 2012	Genome-wide association screening identified DENND1A and other unsuspected PCOS susceptibility loci in Han Chinese	Chen & Shi
2014	DENND1A splice variant (V2) discovered to account for theca cell phenotype in hyperandrogenic oligo-anovulatory PCOS	McAllister
2015	International pediatric endocrinology consensus criteria developed for diagnosis of adolescent PCOS	Witchel

2044

2045

Table 3. PCOS Diagnostic Criteria (see text).					
Diagnostic Parameter (Otherwise Unexplained):	Adult Rotterdam Phenotype A (Classic)	Adult Rotterdam Phenotype B (NIH criteria)	Adult Rotterdam Phenotype C (Ovulatory)	Adult Rotterdam Phenotype D (Non-hyper-androgenic)	Adolescent
Hyperandrogenism †	X	X	X		X

Oligo-amenorrhea	X	X		X	X*
Polycystic ovary	X		X	X	
† Clinical or biochemical evidence * Age- and stage-adjusted; persistent					

2046

2047 **Legends**

2048

2049 **Figure 1.** The reticulum of the adrenocortical zones. ZG: zona glomerulosa,
2050 merging into zona fasciculata. ZF: zona fasciculata. ZR: zona reticularis. Figure
2051 lettering: S is large septum running from the capsule to the ZR, other lettering
2052 delineates space occupied by zona glomerulosa column and cells. Submitted at
2053 original size.

2054 *Reproduced from: Flint JM. The blood vessels, angiogenesis, organogenesis,*
2055 *reticulum, and histology of the adrenal. The John's Hopkins Hospital Reports*
2056 *1900;9:153-230*

2057

2058 **Figure 2.** The anatomy of the female reproductive system drawn by Andreas
2059 Vesalius, 1553. *Reproduced from Andreas Vasalius, De Humani Corporis*
2060 *Fabrica, Sextus.*

2061

2062 **Figure 3.** Major steroid hormones produced by the adult adrenal cortices and the
2063 ovaries. Layout is according to the general biosynthetic pathway from cholesterol.
2064 Enzyme expression patterns are specific to each adrenocortical zone and to the
2065 ovarian theca and granulosa cells, as discussed in text. Conventional numbering of
2066 carbon atoms and lettering of steroid rings illustrated for cholesterol. The top row is
2067 the pathway to progesterone and mineralocorticoids, the second row to
2068 glucocorticoids, the third row to 17-ketosteroids, the fourth row to 17 β -
2069 hydroxysteroids. The dotted 17,20-lyase pathways are probably minor. The

steroidogenic enzymes are italicized. Designations and abbreviations for enzymes according to Miller and Auchus are indicated in the side panel in approximate order of appearance. *Modified from Rosenfield RL, Lucky AW, Allen TD (1980). The diagnosis and management of intersex. Curr Prob in Pediatr 10:1-66 according to Rosenfield RL and Ehrmann DA (2016). The pathogenesis of polycystic ovary syndrome (PCOS): the hypothesis of PCOS as functional ovarian hyperandrogenism revisited. Endocrine Reviews 2016;37:467-520*

Figure 4. A wedge section of a polycystic ovary “almost as large as fundus”, as published in 1935 by Stein and Leventhal. Bar added to indicate 5mm. *Reproduced and modified with permission from: Stein IF, Leventhal ML. Amenorrhea associated with bilateral polycystic ovaries. American journal of obstetrics and gynecology 1935;29:181-9.*

Figure 5. The structure of Searle’s and Syntex’s first generation of synthetic progestins and estrogens compared to the natural hormones progesterone and estradiol. The progestin norethynodrel and estrogen mestranol were the components of the first combined oral contraceptive, Enovid.

Figure 6. Annual PubMed citations of “polycystic ovary syndrome”, 1965-2022.

Figure 7. Changes in adrenocortical steroidogenic gene expression during adrenarchal growth and development of the zona reticularis. The zona reticularis of the adrenal cortex is normally established as a distinct, continuous zone after 3 years of age, is well established by 8 to 9 years of age, and continues to grow and develop until early adulthood. The characteristic changes in the level of expression of differentially expressed key genes in each of the adrenocortical zones is depicted schematically, along with the major secretory product(s) of each zone. Larger and bold fonts indicate that relatively large quantities of the hormone are produced.

* Peripheral tissue 11 β -HSD type 2 converts secreted 11 β -hydroxyandrostenedione to 11-ketoandrostenedione, which is the precursor of most 11-ketotestosterone and, via peripheral tissue 11 β -HSD type 1 activity, 11 β -hydroxytestosterone.

Reproduced and modified by permission from: Rosenfield RL. Normal and Premature Adrenarche. Endocrine Rev. 2021; 42:783 and Auchus RJ, Rosenfield RL. In: Post TW, ed. UpToDate. Waltham, MA: UpToDate, Inc.; 2022:<http://www.uptodate.com>

Figure 8. Estrone hypothesis. This hypothesis proposed that increased LH and LH/FSH ratio resulted from positive feedback on the neuroendocrine system by the excessive acyclic estrone production that arose in part from peripheral conversion of androstenedione in adipose tissue and in part from adrenal secretion due to “exaggerated adrenarche”. Based on concepts proposed by Sam Yen (143).

Figure 9. Two-cell, two-gonadotropin model of human ovarian sex steroid secretion by the small antral follicle, as currently conceived. LH stimulates androgen

2114 formation within theca cells via the steroidogenic pathway common to the gonads
2115 and adrenal glands. FSH regulates estradiol biosynthesis from androgen by
2116 granulosa cells. DENNDA1 is a regulatory protein, the V2 isoform of which was
2117 discovered in 2014 to amplify theca cell steroidogenesis. Androgen formation in
2118 response to LH appears to be modulated primarily by intraovarian feedback at the
2119 levels of 17-hydroxylase and 17, 20-lyase, both of which are successive P450c17
2120 activities. Serum androgen levels do not appear to be tightly regulated: long-loop
2121 negative feedback of estradiol on gonadotropin secretion does not readily suppress
2122 LH at physiologic levels of estradiol and stimulates LH under certain circumstances.
2123 Although androstenedione formation from 17OHP has been demonstrated in ovarian
2124 tissue, human P450c17 activity is very low for this pathway. IL-6 is but one of many
2125 cytokines stimulatory to P450c17 activity. The granulosa cell expression of P450acc
2126 and 3βHSD2 that underlies progesterone secretion by the luteinized follicle is
2127 negligible at this small follicle stage of development. Androgens and estradiol inhibit
2128 (minus signs) and inhibin, insulin, and insulin-like growth factor-I (IGF) stimulate
2129 (plus signs) P450c17activities. Enzyme activities are italicized.

2130 *Reproduced and modified from: Ehrmann DA, Barnes RB, Rosenfield RL. Polycystic*
2131 *ovary syndrome as a form of functional ovarian hyperandrogenism due to*
2132 *dysregulation of androgen secretion. Endocrine Rev 1995;16:322-353 and*
2133 *Rosenfield RL, Ehrmann DA. The pathogenesis of polycystic ovary syndrome (PCOS):*
2134 *the hypothesis of PCOS as functional ovarian hyperandrogenism revisited.*
2135 *Endocrine reviews 2016;37:467-520. Copyright ©2007 and 2016 The Endocrine*
2136 *Society.*

2137

Figure 10. Blood glucose and serum insulin in response to a standard glucose tolerance test in nonobese PCOS (PCO) and control women. Insulin was elevated before and in response to glucose ($p<0.02$), while blood glucose was at similar, indicating insulin resistance. Reproduced from *Chang RJ, Nakamura RM, Judd HL, Kaplan SA. Insulin resistance in nonobese patients with polycystic ovary syndrome. J Clin Endocrinol Metab 1983;57:356-9. Copyright 1983 The Endocrine Society.*

Figure 11. GnRH agonist test results in women with classic PCOS (n=5) vs controls (n=9) during concomitant suppression of adrenal function with dexamethasone. In response to GnRH agonist at 0 hre, PCOS patients had significantly increased early LH responses, followed by a prolonged surge of both gonadotropins peaking at 3-8 hr with FSH baseline and 24-hr area under the curve (AUC) significantly decreased. Ovarian steroid secretion followed with peak responses at 16-24 hr. 17-Hydroxypregnenolone, 17-hydroxyprogesterone (17OHP), androstenedione, estrone, and testosterone (not shown) baseline and maximal responses were significantly greater than those of controls, 17OHP peak responses in PCOS were consistently above those of controls. Thus, there was no evidence of a steroidogenic block, and the results were interpreted as indicating overactive dysregulation of P450c17 activities. * indicates significant difference at time-point, † indicates significant difference in AUC. *Redrawn from data of Barnes RB, Rosenfield RL, Burstein S, Ehrmann DA. Pituitary-ovarian responses to nafarelin testing in the polycystic ovary syndrome. N Engl J Med 1989;320:559-65 and Ehrmann DA, Barnes RB, Rosenfield RL. Polycystic ovary syndrome as a form of functional ovarian hyperandrogenism due to dysregulation of androgen secretion. Endocrine reviews 1995;16:322-53.*

2162

2163 **Figure 12.** Model of mechanisms of functional ovarian hyperandrogenism (FOH)
2164 and PCOS, as currently conceived. Increased intraovarian androgen is responsible
2165 for hyperandrogenemia and follicular maturation arrest, which in turn cause the
2166 cardinal features of PCOS, hirsutism, oligo-anovulation, and polycystic ovaries.
2167 Follicular maturation arrest eventuates in follicular atresia, adding to the androgenic
2168 environment of the ovaries. The cause of the vast majority is dysregulation of
2169 androgen secretion. Since 2014 it is known that abnormal regulation of *DENND1A*
2170 splicing to yield excess of the more active variant DENND1A.V2 causes the typical
2171 type of dysregulated ovarian androgen synthesis in the most severe PCOS
2172 phenotype (phenotype A) and probably accounts for most typical FOH. Obesity-
2173 related elevation of serum insulin and more recently discovered proinflammatory
2174 cytokines also stimulate P450c17 activities seem to account for the FOH of most
2175 obesity. Rare cases of PCOS are secondary to primary virilizing adrenal or ovarian
2176 disorders, severe insulin resistance syndromes, and acromegaly, Primary LH excess
2177 seems to mediate the prenatal androgen programming of FOH.

2178 *Reproduced and modified with permission from Ehrmann DA, Barnes RB, Rosenfield*
2179 *RL. Polycystic ovary syndrome as a form of functional ovarian hyperandrogenism*
2180 *due to dysregulation of androgen secretion. Endocrine reviews 1995;16:322-53.*

2181

2182 **Figure 13.** Transvaginal ultrasounds of an adult polycystic ovary and a normal
2183 ovary. **A.** PCOM in an adult with PCOS. **B.** Normal ovarian morphology in an adult.
2184 OV=ovary volume. FNPS=follicle number per section. Ultrasound images courtesy of
2185 Dr. Maria Lujan.

2186

2187 **Figure 14.** Baseline serum free testosterone levels and ovarian androgenic function
2188 test results in clinically normal, eumenorrheic post-monarchal adolescent (Adol) and
2189 adult female volunteers with normal ovarian morphology in comparison to those
2190 with PCOM and PCOS. Adolescents, 1 yr post-menarcheal to 17.9 yr of age, were
2191 similar to 18-39 yr old adults in each group. Horizontal dotted lines show upper
2192 limits of norma for each test (95th percentiles). **A.** Baseline free testosterone plasma
2193 levels in normal volunteers with normal ovarian morphology (V-NOM) in comparison
2194 to those with PCOM (V-PCOM) and PCOS. PCOM in adolescents has here been
2195 defined as mean ovarian volume >12.0 cc, consistent with 2015 data. V-PCOM had
2196 significantly higher free testosterone than pooled V-NOM (P=0.03). Elevated levels
2197 were found in 2/6 adolescent and 4/30 adult volunteers with PCOM. **B.** SDAST (short
2198 dexamethasone androgen-suppression test) Dexamethasone 0.25 mg/m² orally was
2199 administered at 1200 h, and testosterone was measured 4-hr later. **C.** GnRH agonist
2200 test. Dexamethasone was followed by administration of leuprolide acetate 10 µg/kg
2201 subcutaneously; 17OHP was sampled 20-24-hr later, 4-hr after a repeat 1200-hr
2202 dexamethasone dose. Among the PCOS patients, SDAST was abnormal in 85% (73%
2203 with abnormal GnRHag test), GnRHag test in 66% (92.5% with abnormal SDAST),
2204 Among volunteers with PCOM, 4/6 adolescents and 8/30 adults, including all with
2205 baseline elevation of free testosterone, had either an abnormal SDAST or GnRHag
2206 test result that is in the lower PCOS range.

2207 Source: Modified with permission from: Rosenfield RL. The diagnosis of polycystic
2208 ovary syndrome in adolescents. *Pediatrics* 2015;136:1154-65.

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Figure 15. Pie charts showing the spectrum of ovarian functional abnormalities in age-matched adolescent and adult volunteer women with PCOM (**A**) and the spectrum of ovarian function in women with PCOS (**B**). **A.** Percent of eumenorrheic, clinically normal volunteers with PCOM (n=28 with full test panel) who had PCOS-related elevated ovarian hormones. "17OHP" designates elevated 17OHP response to GnRH agonist test without associated hyperandrogenemia; 38% of this group had AMH elevation. "Free testost" designates elevated baseline free testosterone (asymptomatic PCOS phenotype C); half of these women had AMH elevation, and all had FOH by either GnRH agonist or dexamethasone suppression test criteria. Data from (269) (271) (275). **B.** The sources of androgen excess in PCOS (n=60), by percent arising from each, alone or in combination. Two-thirds of PCOS have typical functional ovarian hyperandrogenism (T-FOH), characterized by 17OHP hyper-responsiveness to LH. The remainder have functionally atypical PCOS, characterized by heterogeneous sources of androgen production: atypical functional ovarian hyperandrogenism evidence by elevated serum testosterone after adrenal suppression by dexamethasone (A-FOH), functional adrenal hyperandrogenism (FAH), and/or unexplained, in which group excessive adiposity was the only apparent source. Data from (261, 269, 276).

Figure 16. Schematic depiction of AMH function. The transition from the resting primordial to the growing primary follicle stage ("recruitment") is independent of serum gonadotropins and is stimulated by androgen. AMH secreted by the granulosa cells of small growing follicles inhibits recruitment. AMH secretion wanes as gonadotropin-dependence of follicles increases. AMH also inhibits P450c17 and

aromatase activities, which restrains both androgen and estrogen biosynthesis by larger antral follicles. As granulosa cells multiply in an increasingly gonadotropin-dependent manner and follicles grow, estradiol inhibits AMH secretion, confining it to follicles under 9 mm. Increasing gonadotropin-dependence and waning AMH production by growing follicles permit emergence of the estrogen-predominant preovulatory follicle. Dashed arrows indicate key stages in follicular growth and development. Solid arrows with minus sign indicate inhibition by AMH and estradiol. Revised from Rosenfield RL. Current concepts of polycystic ovary syndrome pathogenesis. *Curr Opin Pediatr* 2020;32:698-706.

Figure 17. Photomicrographs of subcutaneous adipose tissue stained for the monocyte lineage marker CD68 showing a “crown-like structure” (CLS), macrophages surrounding a dying PCOS adipocyte. CLSs also stain for the specific anti-inflammatory marker CD11c. Women with PCOS had significantly higher density of CLSs than control women. Reproduced from Huang ZH, Manickam B, Ryvkin V, et al. PCOS is associated with increased CD11c expression and crown-like structures in adipose tissue and increased central abdominal fat depots independent of obesity. *J Clin Endocrinol Metab* 2013;98:E17-24. Copyright The Endocrine Society.

Figure 18. Model of the pathophysiology of hyperandrogenic anovulation in PCOS. **Panel A.** 1) FOH can account for all the cardinal clinical features of the syndrome: hyperandrogenism, oligo-anovulation, and polycystic ovaries. Mature pituitary LH secretion is necessary to sustain the ovarian androgen excess, but LH excess is not necessarily present or sufficient to cause it. **Panel B.** Insulin-resistant

hyperinsulinism and obesity are present in about half of PCOS and aggravate its manifestations. 2) Hyperinsulinism stimulates adipogenesis, exacerbate theca cell FOH, and prematurely luteinizes granulosa cells. 3) Increasing obesity, attributable in part to caloric excess, is associated with increased pro-inflammatory cytokines, many of which aggravate FOH, and also exacerbate insulin resistance. 4) Elevated androgen levels stimulate LH excess by interfering with estrogen-progestin negative feedback. 5) The increased LH further aggravates theca cell androgen production, particularly in the presence of hyperinsulinism, which up-regulates theca cell LH receptors; LH becomes additive to FSH in stimulating estrogen-progesterone production by the luteinized granulosa cells. 6) The increased estrogen-progesterone levels act together with androgen-stimulated inhibin production (not shown) to lower FSH levels.

Source: *Modified with permission from: Rosenfield RL and Ehrmann DA. The Pathogenesis of Polycystic Ovary Syndrome (PCOS): The Hypothesis of PCOS as Functional Ovarian Hyperandrogenism Revisited. Endocrinol Rev 2016; 37: 467-520. Copyright ©2016 The Endocrine Society*

Figure 19. Comparison of enzyme activities in PCOS and control theca cells passaged in long-term culture before and after forskolin stimulation. The two activities of P450c17 (17alpha-hydroxylase, **A**, and 17-20-lyase, **B**) and 3β-HSD activity were significantly increased before (control) and after forskolin stimulation, whereas 17β-HSD activity was not. *Reproduced from Nelson, et al. The biochemical basis for increased testosterone production in theca cells propagated from patients*

2282 with polycystic ovary syndrome. *J Clin Endocrinol Metab* 2001;86:5925-33.

2283 Copyright: The Endocrine Society.

2284

2285 **Figure 20.** Hypothetical relationship of the polycystic morphology-PCOS spectrum

2286 to dosage of *DENND1A* or rare deleterious gene variants and to obesity. About one-

2287 quarter of clinically normal women have PCOM, and about half of these have various

2288 subclinical features of PCOS, including about 5% with subclinical evidence of FOH.

2289 Subtypes have been identified within the PCOS A and B phenotypes that have been

2290 related to the prevalence of apparently deleterious intronic *DENND1A* variants by

2291 Dapas, et al (2020). These subtypes correspond closely to the clinically defined

2292 functionally typical and atypical types of FOH (T-FOH and A-FOH) that we have

2293 identified as underlying PCOS phenotypes A-B. Other than *DENND1A*, gene variants

2294 associated with PCOS have more rarely been linked to the *DENND1A* regulatory

2295 network or AMH/AMH receptor. This figure incorporates the hypothesis that the

2296 same adverse gene variants that underlie PCOS also underlie much of PCOM when

2297 present in small number or potency. The manifestations of gene effects on PCOS

2298 phenotype are magnified by obesity on a spectrum of increasing adiposity. Obesity

2299 effects appear to be mediated by insulin and proinflammatory cytokine excess.

2300

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