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BASIC SCIENCE

Differential Gene Expression in Post-Finasteride Syndrome Patients

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ABSTRACT

Background: An organic etiology underpinning post-finasteride syndrome, a constellation of persistent sexual, neuropsychiatric, and somatic symptoms reported by men exposed to 5-alpha-reductase inhibitors (5ARIs), is debated. Persistent changes in neurosteroid levels or androgen receptor expression have been implicated.

Aim: To determine whether differences in gene expression, especially in relevant biologic pathways, exist between patients reporting post-finasteride syndrome symptoms and healthy controls.

Methods: This was a single center, prospective case-control study taking place between March 2013 and September 2018. Men 18 years and older being evaluated for sexual dysfunction (study) or circumcision (control) were eligible for inclusion. Twenty-six men with a history of 5ARI use reporting symptoms consistent with post-finasteride syndrome were included in the patient group. Twenty-six men consented to inclusion in the control group.

Outcomes: The primary outcome measure is gene expression data for genes affecting neurosteroid levels and androgen receptor activity from penile skin cells.

Results: Gene expression of cells from penile skin samples from twenty-six men of median age 38 years (IQR, 33-42) in the study group was compared with that from twenty-six men of median age 41 years (IQR, 35-62) in the control group (P = .13), with 1,446 genes significantly over-expressed and 2,318 genes significantly under-expressed in study patients. Androgen receptor expression was significantly higher in study patients compared to controls (9.961 vs 9.494, adjusted P value = .01). Serum levels of androgen receptor activity markers 5α-androstanediol (0.950 ng/mL [0.749-1.587] vs 0.949 [0.817-1.337], P = .34) or 3α-androstanedione (3.1 ng/mL [1.925-5.475] vs 6.7 [3.375-11.4], P = .31) revealed no significant differences. No significant differences were found between the number of trinucleotide repeats (21.5 [20-23.75], 22 [19-25], P = .94).

Clinical Implications: In this study we present evidence of gene expression correlating with observed biologic differences in patients with post-finasteride syndrome; providers who prescribe 5ARIs should be aware and advise their patients accordingly.

Strengths & Limitations: Strengths of this study include the evaluation of multiple proposed etiologies for post-finasteride syndrome. The study is also strengthened by the fact that not all data matched the initial hypotheses, qualifying the argument for the existence of PFS. Limitations include potential selection bias arising from more severe phenotypes seeking care; lack of gene expression data prior to 5ARI exposure; lack of non-penile tissue samples supposedly involved; and a lack of mechanistic data to imply causality.

Conclusion: This study is the first to consider and demonstrate gene expression differences in patients with PFS as a potential etiology of sexual dysfunction. **Howell S, Song W, Pastuszak A, et al. Differential Gene Expression in Post-Finasteride Syndrome Patients. J Sex Med 2021;XX:XXX–XXX.**

Key Words: Post-finasteride syndrome; Finasteride; Neurosteroids; Androgen receptor; Sexual dysfunction; Gene expression

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INTRODUCTION

5-alpha reductase inhibitors (5ARIs) prevent the reduction of steroids including testosterone, progesterone, androstenedione, epitestosterone, cortisol, aldosterone, and deoxycorticosterone by various isoforms of the enzyme 5-alpha reductase.[1] Inhibition of testosterone reduction in particular results in a decrease in levels of dihydroxytestosterone (DHT), and can therefore result in higher testosterone levels.[2] This effect explains the utility of 5ARIs in certain androgen-related disorders. Although 5ARIs can be helpful in cases of benign prostatic hyperplasia (BPH) or androgenic alopecia (AGA), they come with several known side effects affecting sexual function, including erectile dysfunction (ED) and low libido.[3] These effects may persist even after cessation of medication.[4]

The full range of consequences of 5AR inhibition are not yet known, and other side effects are increasingly reported. In addition to ED and low libido, sexual effects including penile atrophy, diminished ejaculatory volume and force, and an increased incidence of Peyronie's disease have also been reported.[5-7] Adverse effects outside the sexual domain include physical, neurological and psychiatric concerns. This constellation of symptoms occurring in men who are exposed to 5ARIs and experience these symptoms even after discontinuation of treatment is known as post-finasteride syndrome (PFS).[8] Previously, we catalogued the symptoms reported by patients who took 5ARIs for androgenic alopecia as part of PFS.[9] In addition to penile vascular abnormalities and voiding dysfunction, these included changes in body composition, disturbances in memory and attention, and increased rates of depression and anxiety. Of note, subjective changes in body composition including muscular atrophy and increased fat deposition have not been confirmed by physical examination. Outside of our study population, others have reported possible effects of 5AR inhibition on metabolism (including increased insulin resistance, alteration of fat distribution, and cardiovascular disease) and bone physiology.[10]

To date, the existence and biological basis of PFS have been questioned, with some positing a functional or psychogenic etiology.[11] One study reported persistent sexual symptoms in patients on a placebo treatment, while another noted higher reported rates of side effects in patients who were explicitly educated on them.[12-14] However, there is evidence to support an organic etiology. One possibility is decreased levels of neurosteroids in patients with PFS. One study measured plasma and cerebrospinal fluid levels of neurosteroids, including progesterone, testosterone, and their metabolite hormones, in patients with PFS, finding them to be significantly lower compared to healthy controls even years after discontinuation of 5ARI therapy.[15] While this would explain the emergence of sexual, cognitive, and psychiatric symptoms as well as their long-term persistence, it remains unclear why levels would remain low if 5A reduction was not actively inhibited. In addition, changes to neurosteroid levels alone may not adequately explain other PFS symptoms such as body composition changes and genitourinary issues.

Another proposed etiology for the development of PFS is as a response to androgen deprivation mediated by upregulation in androgen receptor (AR) expression. The AR is expressed in multiple tissues across organ systems including reproductive, genitourinary, nervous, musculoskeletal, cardiovascular, and immune.[16] Given that gene overexpression has been implicated in numerous disease states, AR overexpression in response to an androgen-deficient state may negatively affect multiple tissues throughout the body.[17-18] Upregulation in AR expression in penile skin has been demonstrated in patients with persistent sexual side effects following finasteride discontinuation.[19] Overexpression of AR leading to changes in neurosteroid synthesis and action has been suggested as an explanation for certain behavioral phenotypes in mice.[20] In addition, polymorphisms in CAG (polyglutamine-encoding) repeat length have been inversely associated with transcriptional function.[21] Together, overexpression combined with less functional activity may be responsible for certain PFS symptoms, possible through effects on neurosteroid levels but also through AR-induced tissue toxicity.

RNA microarray studies have been used to assess the conditions of an organism and ascertain whether potential disease states can be attributable to differential gene expression.[22] If we can identify processes that affect neurosteroid levels in a way that explains the findings of lowered levels in CSF; significant differences in AR expression or function; or any other biological processes that are differentially expressed, then the case for a biological etiology of PFS is strong. In the present study we compared gene expression data and parameters relating to AR expression and function between men with a history of 5ARI use and reported PFS symptoms with healthy controls. We hypothesize that gene expression in men with PFS symptoms will demonstrate differential expression compared to healthy controls.

MATERIALS AND METHODS

This is a continuation of our previous prospective case-control study that sought to determine the effects of 5ARIs on men's health.[9] In the present work, we sought to assess whether a biological basis for the constellation of symptoms that characterize PFS may exist. Our study population included 26 men with a history of prior 5ARI use, including finasteride and dutasteride, for androgenic alopecia, as well as 26 controls who had not previously taken 5ARIs and presented for evaluation for circumcision. Sample size was maximized to attain the highest possible power given the rarity of PFS; power calculations using the method described by Orr and Liu for microarray experiments showed power of 0.80 for detecting a fold change of 1 assuming a differentially-expressed gene rate of 1% was met at 10 men.[23]

The study was approved by Institutional Review Board and informed consent was obtained from all the participants prior to the start of the study. Data collection took place at the primary investigator's urology clinic from March 2013 to September 2018. Eligibility criteria included men over 18 years of age who were being seen for sexual dysfunction.

Penile skin biopsy was performed to obtain samples from study patients. Procedures were conducted in the office using a small punch biopsy instrument after local anesthetic injection. The biopsy area was closed using a single 4-0 chromic suture. Patients were directed not to engage in sexual activity for one week following the procedure, and there were no complications arising from this intervention. The biopsy was obtained from the base of the penile shaft at the penoscrotal junction above the scrotum; scrotal skin was not included in the sample. For control patients, the study protocol allowed for either penile skin biopsy as described above or via circumcision to obtain a foreskin sample. All controls who enrolled in the study underwent circumcision.

Total RNA was extracted from penile skin samples using the RNeasy Plus Micro Kit (Qiagen, 74034). The RNA samples were submitted to the institution's Microarray Core Lab for analysis. To pass the quality check, samples required an A260/A280 ratio higher than 2.0 and an A260/A230 ratio higher than 1.7. After the samples passed quality check, they were hybridyzed to microarrays [Agilent Array (8X) chip (Human GE V2 Array, 8 × 60K)] and analyzed. Using the resulting gene list, genes were sorted into two sub-lists, depending on whether they were over- or under-expressed. Genes with a positive $\log_2$ fold change value were considered over-expressed, while those with a negative $\log_2$ fold change value were considered under-expressed. Pathway analysis was performed on genes in each category that were over- or under-expressed to identify likely clusters of function that would be affected. These pathways were then examined for clinical relevance and further investigated to determine specific alteration in the study group patients.

Differential expression analysis was performed using a moderated Limma t-test in the standard fashion for microarray experiments [http://bioconductor.org].[24,25] Data were background-corrected using the 'normexp' method with an offset of 16 added to the intensities. Background-corrected data were then log2-transformed and quantile-normalized. Moderated t-statistics were used to test if genes were differentially expressed between the study and control groups. Benjamini-Hochberg method was used to estimate false discovery rate (FDR). FDR < 0.05 was considered statistically significant.

Pathway analysis and process enrichment analysis was performed using Metascape for both gene sets [http://metascape.org],[26] The enrichment background contained all genes in the human genome. Terms, or biologic processes, were grouped into clusters based on similarities in the genes each process contains. The most statistically significant term within a cluster is chosen to represent the cluster. Cluster significance is demonstrated by multi-test adjusted p values reported as q values with log10 transformation. The top twenty most significant clusters, were further evaluated for clinical relevance. Several were excluded: those that referred to processes involved in early development of organs that would not explain PFS development in adulthood, such as the heart and renal system; those described by terms too vague to meaningfully analyze; and those which imply an effect on gene expression itself, which is interesting to consider, but out of the scope of this study.

AR expression was assessed using the above process. The number of CAG repeats was determined and compared between groups to evaluate whether a CAG repeat polymorphism might contribute to the development of PFS symptoms. Two metabolites of testosterone, 5-alpha-androstanediol and 3-alpha-androstanediol glucuronide, were also measured and compared to evaluate for any disturbances in androgen metabolism.[27] Continuous variables were evaluated using Welch's t-test to avoid assuming equal variance with a P value of $< .05$ considered statistically significant. Microsoft Excel (version 365; Microsoft Corporation, Redmond, Washington) was used for all statistical analyses not performed with Bioconductor as previously described.

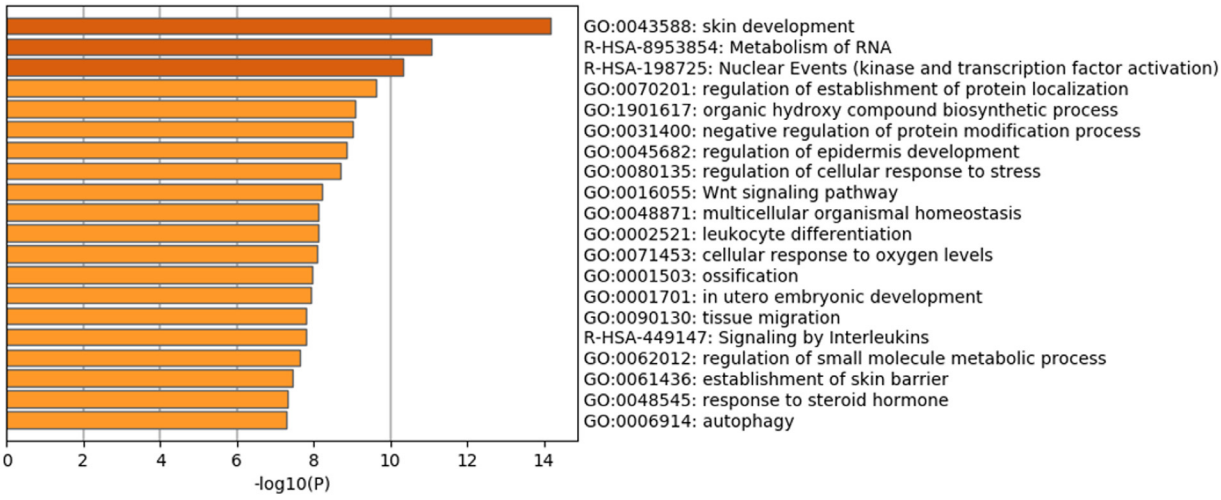

Figure 1. Top 20 significant clusters among over-expressed genes.

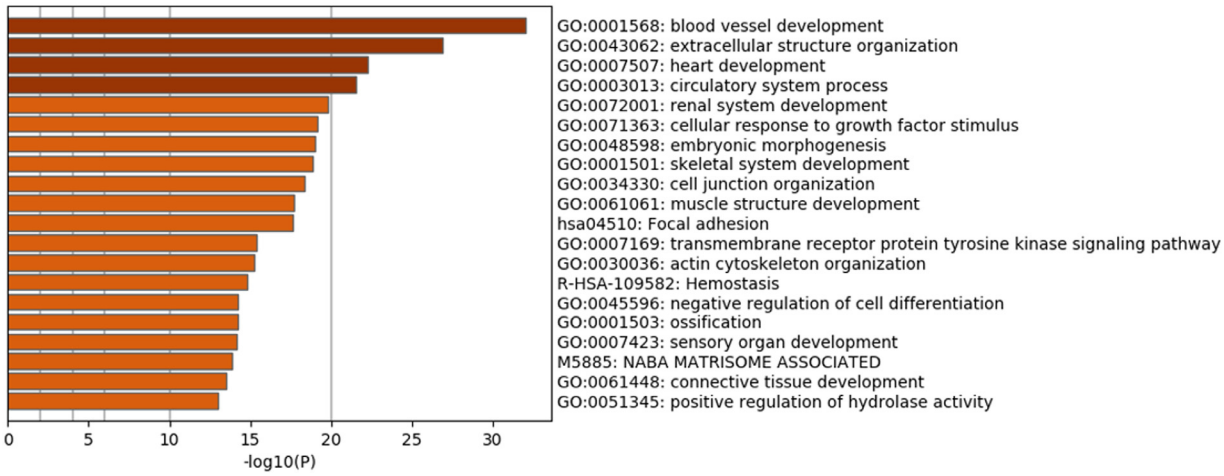

Figure 2. Top 20 significant clusters among under-expressed genes.

RESULTS

Baseline Characteristics

Baseline characteristics are the same as in our prior work and reported here for completeness in Table 1.[9] Median treatment duration with finasteride was 18 months (IQR, 4-96 months). Eleven subjects used 1 mg of finasteride (0.2-1.25 mg). One subject used 0.5 mg of dutasteride for 24 months after discontinuation of finasteride (which had been taken for 96 months in this patient). In study patients, median total testosterone level was 450 ng/dL (IQR, 373−558 ng/dL), while median DHT value was 366 ng/dL (IQR, 373−509 ng/dL). Testosterone and DHT levels were available only for a small number of controls, so comparison could not be performed. Median age of study patients was 38 years (IQR, 33-42), with median age of controls being 41 years (IQR, 35-62; P = .13). Median body mass index (BMI) was 24.5 kg/m2 (IQR, 22.1−25.8 kg/m2) for study patients and 30.6 (IQR, 27.1−33.3) for controls (P < .001). Groups did not significantly differ by age, although controls had significantly higher BMI.

Table 1. Comparison of baseline characteristics between PFS and control groups

Variable	5ARI Group (n = 26)	Controls (n = 26)	*P*
Age (years)	38 [33-42]	41 [35-62]	.13
Duration of 5ARI use (months)	18 [4-96]	0 [0-0]	-
Duration of time off 5ARI at biopsy (years)	6 [3-10]	-	-
BMI (kg/m^2)	24.5 [221.-25.8]	30.6 [27.1-33.3]	< .001
Testosterone (ng/dL)	450 [373-558]	315 [249-319]	-
DHT (ng/dL)	366 [373-509]	-	-
Hemoglobin (g/dL)	15.9 [15.2-16.3]	14.9 [14.3-15.5]	.07

Results displayed as median [interquartile range]. Testosterone levels were only available for 3 controls, so there were too few observations to calculate a *P* value. DHT levels were not available for any controls.

Comorbidity Profiles

Sexual, genitourinary, psychiatric, and anti-androgenic comorbidities were evaluated in this population and reported in our previous work.[9] These are summarized in Table 2. Briefly, significant differences in validated questionnaires including International Index of Erectile Function (IIEF), International Prostate Symptom Score (IPSS), and the nine-item Patient Health Questionnaire (PHQ-9) were observed, with differences in response pattern to Androgen Deficiency in the Aging Male (ADAM) as well. Median total IIEF score was 35 (IQR, 29-43) for study patients and 29 (IQR, 27-32) for controls. Median total IPSS score for study

Table 2. Sexual, genitourinary, psychiatric, and anti-androgenic comorbidity profiles between PFS and control groups

Questionnaire	5ARI Group (n_{PFS})	Controls (n_c)	*P*
IIEF (n_{PFS}=23, n_c=14)	35 [29-43]	29 [27-32]	.035
IPSS (n_{PFS}=23, n_c=15)	10 [5−16]	3 [2−8]	.009
PHQ-9	10 [6.5−16]	1 [0−2]	< .001
ADAM**			
"Yes" to 3+ items?	Yes (1-3, 5-7, 10)	No	-
"Yes" to item #1?	Yes	No	-
"Yes" to item #7?	Yes	Yes	-

Results displayed as median [interquartile range].
**Any of these criteria may indicate hypogonadism.

patients was 10 (IQR, 5−16) compared to 3 (IQR, 2−8) for controls. The study group had a median total PHQ-9 score of 10 (IQR, 6.5-16) compared with 1 (IQR, 0-2) in the control arm. In addition, study patients on average met all three criteria to suggest hypogonadism according to the ADAM questionnaire, while controls met only one. Finally, study patients reported a high number of subjective genital and musculoskeletal complaints compared to their counterparts in the control group.

Microarray Analysis

Penile skin samples from 52 patients yielded analysis of 46,204 gene symbols. After false discovery rate correction, 1,446 genes were significantly over-expressed and 2,318 genes significantly under-expressed in study patients. Fold change for over-expressed genes ranged from 1.105 to 5.453, while for under-expressed genes it ranged from -1.095 to -11.236. Of particular interest, we found AR expression to be significantly higher in study patients compared to controls (adjusted *P* value = .01).

Pathway Analysis

Pathway analysis on both the over- and under-expressed gene sets yielded fourteen and fifteen clinically relevant clusters, shown in Figure 1 and Figure 2, respectively. Of note, the levels of significance are much higher for the under-expressed genes, indicating it is more likely that these biologic systems are affected by differential gene expression in our study population. As we present these results, we will refer to clusters derived from the over-expressed gene set as "up-regulated," while clusters derived from the under-expressed gene set will be termed "down-regulated."

Sexual Effects: Penile Vascular and Soft Tissue Health

We previously reported that the majority of our study patients suffered from confirmed penile vascular abnormalities on penile Doppler ultrasound, including arterial insufficiency, possible ED, and venous leak. In this study, we found that pathways including tissue migration, angiogenesis, and vasculature development were up-regulated. Conversely, pathways including blood vessel development, angiogenesis, and positive regulation of epithelial and endothelial cell migration and proliferation were down-regulated.

In addition to vascular issues, soft tissue components of penile anatomy may be affected in PFS. Peyronie's disease, along with changes in penile length and testicular size, are reported in patients who have taken 5ARI. We found that pathways involving extracellular matrix regulation (including the "matrisome"), cell junction organization, and connective tissue health were down-regulated.

Genitourinary Effects: Voiding

We previously presented evidence that immune system involvement and inflammation, bolstered by the low DHT-environment seen in 5ARI use, may lead to development of benign prostatic hyperplasia and subsequent voiding symptoms in PFS. In this study, we observed that pathways controlling T-cell development, proliferation, and function, along with pathways involving cytokine signaling, were upregulated in study patients.

Neurological Effects: Neuro-steroids

Although neuro-steroids levels should return to normal following discontinuation of 5ARI, we identified persistent changes in gene expression in study group patients that may explain the preservation of changes in levels of progesterone, testosterone, and their metabolites reported by Caruso et al.[15] Upregulated clusters include "organic hydroxy compound biosynthetic process," "regulation of small molecule metabolic process," and response to steroid hormone. Processes involving regulation of cholesterol synthesis were particularly involved. Pathway analysis did not reveal a significantly down-regulated pathway involved in steroid metabolism or regulation.

Neurological Effects: Nervous Tissue Maintenance

Separately from the effects of neuro-hormones, processes controlling nerve cell health appear to be impacted by 5ARI exposure. An upregulated cluster identified as "kinase and transcription activation" further specified as involving processes controlling neurogenesis and immune responses, are upregulated in study patients. However, transforming growth factor beta and bone morphogenic protein signaling, along with several pathways including neuron differentiation, neurogenesis, and axonogenesis, are pathways that are down-regulated. In addition, the actin cytoskeleton organization cluster was down-regulated. Notably, down-regulated pathways exhibit much higher levels of significance.

Physical Effects: Musculoskeletal and Metabolic Changes

Many of our patients reported musculoskeletal complaints, including fatigue, muscle atrophy, and joint pain, along with skin changes and visual disturbances. Pathway analysis revealed upregulation in pathways affecting skin development, epidermis development, and "establishment of the skin barrier". Ossification was also upregulated. Down-regulated clusters included skeletal system development, muscle structure development, sensory organ development, sensory organ (visual) development, and connective tissue development.

Altered steroid metabolism can affect metabolic health, and may lead to issues with insulin resistance, increased fat deposition, and cardiovascular disease.[10] We observed upregulation in pathways affecting insulin (within a cluster called "regulation of establishment of protein localization") including positive regulation of peptide secretion, regulation of insulin secretion, and response to carbohydrate. Similarly, we observed upregulation in pathways controlling homeostasis and responses to stress, which

Table 3. Parameters related to AR expression and function

Variable	5ARI Group	Controls	Fold Change	*P*
AR expression	9.961	9.495	1.381	.01
CAG trinucleotide repeats	21.5 [20-23.75]	22 [19-25]	-	.94
5α-androstanediol (ng/mL)	0.950 [0.749-1.587]	0.949 [0.817-1.337]	-	.34
3α-androstanedione glucuronide (ng/mL)	3.1 [1.925-5.475]	6.7 [3.375-11.4]	-	.31

Unless otherwise noted, results displayed as median [interquartile range]. AR expression is given as log2-transformed and quantile-normalized means. The *P* value for AR expression is a Benjamini-Hochberg adjusted value.

both were found to be related to metabolic processes on further analysis of relevant annotations. Down-regulated clusters included circulatory system process (involving processes like cardiac muscle contraction and ion transport); "cellular response to growth factor stimulus" specifically referring to the TGF-beta signaling pathways involving bone morphogenic protein, which regulate osteogenesis, growth, and homeostasis;[28] and "transmembrane receptor protein tyrosine kinase signaling pathway," which included pathways involved in the cellular response to insulin.

Androgen Receptor

Data evaluating AR parameters is reported in Table 3. Per microarray data, *AR* was overexpressed in study patients. However, neither the number of CAG repeats nor the levels of 5-alpha-androstanediol and 3-alpha-androstanedione glucuronide were significantly different between study and control groups. For study patients, the median length of CAG repeats was 21.5 (IQR, 20-23.75), while for controls the median length was 22 (IQR, 19-25). The median level of 5-alpha-androstenediol was 0.950 ng/ml (IQR, 0.749-1.587 ng/ml) in study patients and 0.945 ng/ml (IQR, 0.817-1.337 ng/ml) in controls. The median level of 3-alpha-androstanedione glucuronide was 3.1 ng/ml (IQR, 1.925-5.475 ng/ml) in study patients and 6.7 ng/ml (IQR, 3.375-11.4 ng/ml) in controls.

DISCUSSION

In the present study, we explored the hypothesis that PFS is underpinned by a biological mechanism triggered by use of 5ARIs and the resultant androgen-deficient state. We identified genes that are differentially expressed in penile skin tissue between men with a history of 5ARI use and PFS symptoms and healthy controls, identifying biological pathways that were up-regulated or inhibited and may be relevant in the development of PFS symptoms. We proposed that these effects are mediated largely through effects on neurosteroid levels or changes in AR expression leading to down-stream effects on sexual, physical, neurological, and psychiatric functioning. In addition, we identified a number of other pathways involving differentially expressed genes that may also contribute to PFS symptoms.

Neurosteroids

When 5A-reduced steroids such as DHT are 3A-reduced, they become 3-alpha,5-alpha neurosteroids. These molecules are steroid hormone metabolites that effect inhibitory or excitatory function in the central nervous system through action on neuronal membrane receptors.[29] In addition, they can modify gene expression through interaction with intracellular steroid receptors in nervous tissue.[30] They play a role in in several important functions including mental health (depression, anxiety, stress), cognition (learning, memory), and nervous system plasticity.[31-35] Both inhibitory and excitatory neurosteroids ultimately have antidepressant, anxiolytic, neuroprotective, neurogenic, and cognition-enhancing effects.[36-37] 5ARIs may induce cognitive and psychiatric side effects through decreased neurosteroid levels by preventing steroid hormones from metabolizing through reduction.

We identified genes involved in pathways that may affect neurosteroid levels which were upregulated. Considered alone, this would appear to be at odds with the findings reported by Caruso et al.[15] However, it is possible that this particular alteration in gene expression may represent a feedback loop attempting to drive synthesis to replace depleted hormone levels, including progesterone, testosterone, and their reduced neuro-active metabolites, in response to 5AR inhibition. However, to better understand the likely effect of 5ARI exposure in our population of PFS patients, we searched for other gene expression changes that could further elucidate the mechanisms involved. We considered three ways for neurosteroid action to be affected: (i) changes in pathways affecting steroid hormone metabolism; (ii) changes in pathways affecting neuronal membrane receptors; and (iii) changes in pathways affecting the androgen receptor, which we found to be significantly over-expressed in PFS patients. Using a list of genes of interest across these categories that we constructed prior to conducting the experiment, we cross-referenced our microarray results to identify whether genes involved with these receptors were differentially expressed in our study population.

Steroid hormones are both precursors for neurosteroids and have their own functions; biological pathways that affect neurosteroids likely affect steroid hormones, and vice versa. Thus we considered whether specific genes relating to steroid synthesis and function were altered in PFS patients. For genes related to steroid metabolism, specifically aldosterone, corticosterone, and

cortisol, we identified several differentially expressed genes. For aldosterone: a gene coding for *BMP2*, which can inhibit aldosterone biosynthesis, was over-expressed.[38] At the same time, *BMP6,* whose product can positively regulate aldosterone secretion, was under-expressed.[39] Knowing that aldosterone is a substrate of 5AR, a decrease in available levels for reduction to its neuro-steroid (3-alpha,5-alpha-tetrahydroaldesterone) compounded with active inhibition of this process could impair neuro-steroid activity significantly. For corticosterone and cortisol, only one gene, *PTPN11*, which negatively regulates cortisol secretion, was identified. Mutations in this gene can lead to genetic syndromes, and it has also been implicated in several cancers.[40-42] In our microarray analysis, *PTPN11* was under-expressed, implying a lack of negative regulation and thus increased cortisol secretion. Another gene, *TAC1,* positively regulates corticosterone secretion.[43] Several genes that play a role in the response to cortisol and corticosterone were upregulated; notable genes include those playing a role in inflammation, such as *FOS, IL1RN, PTGS2, SDC1,* and *TNF*, as well as *ZFP36* which upregulates tumor necrosis factor secretion.[44-49] Thus, while aldosterone action seems to be decreased in PFS patients, cortisol and corticosterone, which affect diverse processes but are specifically known to be elevated in stress states, are increased. Given that genes that drive the inflammatory effects of steroid hormones are upregulated, it is possible that this chronic state of inflammation may be contributing to PFS. Furthermore, several genes that respond to cortisol that are involved in processes including cardioprotection (*FIBIN, UCN*); bone mineralization (*SPARC*); and normal brain development (*CASP3*) were under-expressed.[50-53] Deficiency of another under-expressed gene, *CPS1*, has been shown to increase levels of ammonia in the blood.[54] The under-expression of these "protective" genes in the context of increased cortisol levels combined with over-expression of inflammatory regulators portrays an emergent picture of chronic stress leading to damage to diverse body systems including the circulatory, skeletal, and nervous systems.

We identified several differentially regulated pathways that may further support the indications of stress as a contributor to PFS. Upregulation in pathways affecting insulin (within a cluster called "regulation of establishment of protein localization") including positive regulation of peptide secretion, regulation of insulin secretion, and response to carbohydrate, may contribute to insulin resistance along with the effects of cortisol.[55] Upregulated pathways controlling homeostasis and responses to stress specifically related to metabolic processes also support the existence of a chronic stress state. Relevant down-regulated clusters affecting cardiac muscle contraction, homeostasis, and the cellular response to insulin, may be further contributing to physical symptoms in PFS because their relative absence worsens the inflammatory and metabolic damage wrought by increased cortisol and upregulated inflammation.

Inhibitory neurosteroids prevent neuro-transmission by acting as positive allosteric modulators of the $GABA_A$ receptor.[36] Excitatory neurosteroids increase neuro-transmission by inhibiting the $GABA_A$ receptor, weakly activating the NMDA receptor, and through agonist activity at the sigma receptor.[36] For genes related to neuronal membrane receptors, we found that no genes affecting $GABA_A$ or sigma receptors were differentially expressed. However, several genes affecting NMDA receptors were upregulated (*APOE, GRIN2A, TIAM1*) while one (*SHANK3*) was under-expressed. *APOE* codes for apolipoprotein E, a structural component of plasma lipoproteins that plays a role in cholesterol homeostasis and differentially affects NMDA receptor expression depending on the allele.[56] *GRIN2A* codes for a subunit of the NMDA receptor required for normal neurologic function; alterations in this gene are known to cause neurodevelopmental and seizure disorders.[57] *TIAM1* codes for a protein required for NMDA receptor function to regulate neuron development.[58] *SHANK3* codes for a structural protein in glutamatergic synapses, which, if deficient, can lead to decreased function of NMDA receptors through an actin intermediary.[59] Taken together, over-expression of *APOE, GRIN2A,* and *TIAM1* likely results in increased function of the NMDA receptor and increased nervous system development through neurogenesis. However, concomitant under-expression of *SHANK3* may interfere with this process at a downstream level that negates whatever higher level of activity is generated by the over-expressed genes. When we also consider the down-regulated actin cytoskeleton organization cluster from our pathway analysis, it is possible that NMDA receptor function is decreased, which potentially explains the cognitive deficits reported in PFS.

Androgen Receptor

The *AR* itself was over-expressed in PFS patients. Overexpression of normal genes has diverse pathologic consequences across a variety of tissues, leading to certain neurodegenerative diseases, fibrosis, diabetes, and cancer.[17] Since patients in this study did not have an elongated CAG polymorphism, we can consider their *AR* gene to be normal. Overexpression of *AR* in penile tissue may be responsible for sexual symptoms experienced by PFS patients.[19] If *AR* is also overexpressed in other tissues, for example nervous tissue, it may play a role in cognitive and psychiatric symptoms as well.

AR expression is not the only relevant factor: in addition, we must consider actual AR activity. We hypothesized that significant differences in CAG trinucleotide repeats leading to AR resistance to androgen signaling through decreased transcriptional activity may lead to PFS symptoms. However, we found that there was no significant difference in the length of the polyglutamine-encoding stretch between PFS patients and controls. In addition, given the levels of androgen metabolites did not differ significantly between groups, we cannot attribute symptom development in patients to differential AR activity alone. However, AR function is complex and involves many genes separate from *AR*. Many such genes of interest were identified in the microarray analysis.

For other genes involved in AR signaling, we identified fifteen over-expressed and twelve under-expressed genes. Several key points may be gathered from this data. First, several genes coding for 17-beta-hydroxysteroid dehydrogenases, enzymes involved in steroid conversion, were under-expressed.[60] Specific isoforms included 6 (which also has 3-alpha reductase activity and catalyzes conversion of androstanediol into DHT in the prostate); 7 (involved in cholesterol metabolism and the reverse process of 6); and 11 (which likely plays a role in neurosteroid synthesis).[61-62] Isoform 4, the only one to be over-expressed, is involved in estrogen metabolism in the uterus, so its relevance to PFS is unclear.[63] In addition, the 3-beta-hydroxysteroid dehydrogenase isoform 7, important for steroid hormone synthesis, was under-expressed.[64] Together, this provides a potential mechanism for persistent cognitive and psychiatric symptoms in PFS, as inhibition of neuro-steroid action is continued independently of active 5AR inhibition by medication. Second, several genes acting as negative regulators of AR activity were over-expressed, while others, which increase or otherwise modulate AR activity were under-expressed. Over-expressed genes included *PIAS2* and *FOXP1*, both of which function to negatively regulate the AR signaling pathway.[65] Under-expressed genes included *TGFB1I1* (also known as ARA55), *DAXX, TAF1*, and *PARP1*, which function in transcription and regulation of DNA repair and apoptosis.[66-69] This combined effect may result in reduced AR function as a transcription regulator. Third, several genes acting as positive regulators of AR activity or involved in AR function were over-expressed, including *BUD31, RNF4, RNF6, DDX5,* and *HDAC6.*[70-74]

Depending on the interaction with the previously discussed genes, AR activity could be increased, decreased, or balanced out in PFS patients. If it is higher than in controls, this may be a result of the body attempting to correct for the decreased level of neurosteroids available. If it is lower than in controls, it could be another contributor to anti-androgenic symptoms in PFS. The fact that AR expression was elevated in PFS patients suggests a chronic androgen-deficient, or activity-deficient, state. However, the lack of a difference in androgen activity markers indicates the opposite conclusion. Since expression data came from specific tissue, it is possible that there are differing site-specific expression patterns in patients. Ultimately, whether the differentially-expressed genes affecting AR activity play a role in PFS remains unclear given the lack of mechanistic data, and requires investigation pointed in this direction.

Other Contributing Pathways

In addition to the effects of decreased neurosteroid activity, changes to steroid levels, and AR overexpression, it is possible that changes to other biologic mechanisms may be present in PFS patients which contribute to the syndrome. Results of pathway analysis indicated involvement of biological processes affecting the sexual, genitourinary, neurological, musculoskeletal, cardiovascular, metabolic, and immune systems. From our data, it is unclear to what extent neurosteroids and AR are responsible for changes in expression for genes affecting these pathways.

We previously established the existence of persistent sexual and genitourinary symptoms using validated questionnaires that showed significant differences between PFS patients and healthy controls. We found significant differences in total IIEF scores as well as sexual desire and overall satisfaction domains, along with significant issues with incomplete emptying, frequency, weak stream, and overall quality of life per the IPSS. In our study, we have already established that low levels of neurosteroids may contribute to sexual dysfunction in PFS, but our finding that many PFS patients suffer from penile vascular abnormalities as well implies that other processes may be at play. We found pathways controlling vascular remodeling and development to be both upregulated and downregulated, with the downregulated pathways more significantly enriched. Most likely, this points to an issue with dysregulated vascular development leading to abnormalities that could contribute to poor penile erectile function. In addition, Peyronie's disease has been observed in the context of 5ARI treatment and is thought to be due to decreased DHT activity. However, our finding that pathways controlling extracellular matrix function are down-regulated may also be contributing to penile soft tissue abnormalities. Finally, given that immune system process are thought to contribute to BPH, the combination of upregulated immune pathways including T-cell development and cytokine signaling as well as reduced androgenic function explains voiding dysfunction in PFS patients. Furthermore, the upregulated inflammatory processes may be contributing to a chronic stress state beyond their effects on the genitourinary system.

Our earlier work noted numerous somatic and physical complaints as well as a significantly higher body mass index in PFS patients. Complaints included generalized fatigue and weight changes along with musculoskeletal issues (subjective muscle atrophy, back pain, joint pain), skin changes (xeroderma), issues with vision (ocular hyperhidrosis, disturbances, and xerophthalmia). We observed upregulated pathways affecting skin and epidermis development, potentially leading to perceived dryness and discomfort of the skin. Other relevant processes including pathways affecting skeletal system, muscle structure, and connective tissue (specifically cartilage) development were downregulated, potentially explaining the musculoskeletal symptoms. Visual sensory development was also downregulated. Other symptoms including palpitations, cold flashes, and rapid aging may be attributable to the underlying chronic stress state we believe is a result of increased cortisol and inflammatory gene over-expression. Polydipsia may be result from under-expression of the aquaporin-1 channel, which is normally upregulated by cortisol, driving a thirst response.[75]

Other pathways affecting nerve cell development were observed to be differentially regulated, though it is unclear what the overall effect may be. The upregulated "kinase and transcription activation" cluster included NTRK and NGF signaling pathways, which control processes including memory, pain

sensation, neural plasticity, synapse signaling, and mood stabilization.[76-77] This particular set of processes may account for the fact that cognitive symptoms are not always found to be persistent according to validated questionnaires.[78] However, other studies have found persistent cognitive deficits, which is likely driven by the downregulated pathways that otherwise promote neuron differentiation and nervous cell genesis, such as the transforming growth factor-beta and/or bone morphogenic protein signaling pathways. Deregulation in these pathways has been implicated in a number of cognitive and neurodegenerative disorders.[79] Ultimately, the specific balance resulting from the complex interplay of neurosteroids, neurotransmitter receptors, and nervous tissue developmental regulators in each individual patient may explain why some men develop persistent cognitive symptoms while others do not.

Our data revealed differential expression of many genes in pathways controlling gene expression itself. This may be due in part to the fact that steroid hormones act by inducing changes in gene expression or by AR activity, and may also explain the nature of the persistent effects of 5ARI exposure. Namely, exposure to 5ARI could lead to permanent changes in genetic expression through unknown mechanisms which cause the changes we were able to measure and analyze. However, further work would need to be done to explore these mechanisms, and is beyond the scope of the current study.

Strengths and Weakness

There are several strengths to this study, including the use of advanced gene expression and pathway analysis and gene expression data from a large cohort of cases and controls. This study is the first to evaluate multiple proposed etiologies for PFS and provide biologic evidence for each. In addition, this study is strengthened by the fact that not all data matched the initial hypothesis, qualifying the argument for existence of PFS as a unified clinical entity. This study also has weaknesses and limitations. We base the objective existence of symptoms partially on subjective survey data. Selection bias may arise from only the more severe phenotypes seeking care. Gene expression data prior to 5ARI exposure is unavailable for comparison and thus we are less certain that differences in expression between study and control groups are attributable to 5ARI exposure. Further, we lack tissue samples from supposedly involved tissues (ie CNS tissues), ultimately limiting the conclusions that can be drawn from both the microarray and AR expression data. Finally, there exists a lack of mechanistic data to imply causality for proposed mechanisms; gene expression changes alone do not necessarily translate to concordant changes in protein expression or protein activity.

CONCLUSION

This study is the first to consider gene expression differences in men with PFS in explaining the etiology of this condition. Given gene expression per se is not mechanistic and does not imply causality, experiments with downstream processes of protein expression and activity should be undertaken to provide mechanistic data and clarify the results of this work. Further investigation should also explore upstream processes including the mechanisms regulating gene expression in the setting of PFS and identify risk factors for individuals, with a potential focus on genetic risk factors. At this time, patients should be informed regarding possible side effects of 5ARI that may persist even following discontinuation of treatment as part of their counseling.

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STATEMENT OF AUTHORSHIP

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