**Study Synopsis**

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| Study Title | Detection of Testosterone Microdosing in Women |
| Objectives | To evaluate the sensitivity and relative efficacy of serum and urine steroid and hematological profiles to detect testosterone microdosing in women |
| Study drugs | Testosterone gel 1% (Testogel, ARTG 227563) for transdermal use  |
| Dose and administration | Testosterone 12.5 mg (1 pump actuation comprising 1.25 g gel) daily for 7 days |
| Study design | Single centre, open label study of transdermal testosterone gel with run-in, treatment and run-off (post-treatment) phases |
| Endpoints | Primary: Serum (LC-MS) and urine (GC-MS) steroid profiles including carbon isotope ratio (CIR) of testosterone and its 5α reduced metabolites (DHT, epiandrosterone sulphate) and hematological profile (full blood count including hemoglobin and reticulocytes) before, during and after testosterone administrationSecondary: Dried blood spot serum, serum LH and FSH and urine LH (immunoassay), UGT2B17 genotype, Safety: hematology (full blood count) and biochemistry (liver and renal function) |
| Sample size | 12 healthy women not using hormonal drugs other than hormonal contraception |
| Entry Criteria | **Inclusion:** * Healthy women aged between 18 and 60 years
* Provide written, informed consent and willing to comply with all study requirements

**Exclusion:** * Pregnancy, breast feeding or seeking fertility within next 6 months
* Using hormonal drugs other than oral contraception (eg estrogen replacement)
* Athletes subject to regular urine drug testing
* Contraindication to testosterone (breast cancer, polycythemia)
* Major, serious or chronic medical disorders including chronic viral (HIV, hepatitis) infection that require regular prescribed medication
* Severe or extensive skin disease that interferes with transdermal drug delivery
* Regular medications that interfere with dermal absorption or metabolism of testosterone
* History of androgen or other drug abuse within last year
* History of major psychiatric disease or psychological condition that may limit understanding and compliance with study requirements in the investigator’s opinion

**Precaution:*** Working in an occupation that requires urine drug testing
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| Study procedure | Each eligible, consenting participant will be studied for at least 2 weeks before, during 7 days of treatment and for 3 weeks after completion of treatment Participants will have the testosterone gel provided to them without cost  |
| Study visits and procedures | A total of 12 visits comprising a Screening visit which will provide a standardized medical history and physical examination (to determine eligibility and consent) followed by 11 additional clinic visits over 5 weeks for eligible consenting participants. Eligible participants will then provide blood and urine samples at 5 run-in visits over at least 2 weeks prior to the start of testosterone treatment. Together with the immediate pre-treatment samples, this will provide a total of 6 sets of blood and urine samples prior to start of treatment. At the start of treatment, participants will be supplied with the testosterone gel bottle and instructions how to apply the treatment. Treatment period consists of 7 days with daily application of one actuation of the testosterone gel applied to the upper arms, chest or abdomen, using the same anatomical location on alternate sides of the body each day. At the day 7 visit at the end of treatment, the participants will provide blood and urine samples and return the used testosterone gel bottle. Run-off period of 14 days comprises 5 clinic visits at days 1, 2, 4, 7 and 14 after cessation of treatment to provide further blood and urine samples. |
| Statistical analysis | Mixed model linear analysis with serial repeated measurements of serum and urine steroids and hematological profile before, during and after testosterone treatment with age and body weight (or BMI) as covariates. For analytes where there is a significant difference from baseline, linear discriminant analysis and receiver-operated curve (ROC) analysis will be used to define the optimal cutpoint (Youden’s index) for individual and combined parameters to detect testosterone administration as well as the window of detection during run-off.The findings will also be analyzed with ABP software for steroidal and hematological modules.  |
| Principal investigator | David J Handelsman |
| Co-Investigators | Sasha Savkovic, Leo Turner, Glenda Fraser, Carolyn Fennell, Veena Jayadev, Ann J Conway |
| Drug supply | Transdermal testosterone gel (Testogel 1%, Besins Healthcare) |
| Study administration | ANZAC Research Institute |
| Clinical study center | Andrology Department, Concord Hospital |
| Ethics jurisdiction  | SLHD Human Ethics Committee (Concord Hospital) and CTN, TGA |
| Anti-doping laboratory collaborator | Sports Medicine Research and Testing Laboratory, Salt Lake City, Utah, USA |
| Study sponsor | Sydney Local Health District |

**Schedule of Tests**

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Visit** | **V1** | **V2-5** | **V6** | **V7** | **V8** | **V9** | **V10** | **V11** | **V12** |
| **Study Phase** | **Run-in1** | **Treatment** | **Run-off** |  |
| **Day2** | **-14**  | **-13 to -1** | **0** | **7** | **8** | **9** | **11** | **14** | **21** |
| **Screening, eligibility & consent3** | X |  |  |  |  |  |  |  |  |
| **Treatment**  |  |  | Supply | Return |  |  |  |  |  |
| **Blood sample4** | X | X X X X  | X | X | X | X | X | X | X |
| **Blood sample for DNA** | X |  |  |  |  |  |  |  |  |
| **Urine sample5** | X | X X X X  | X | X | X | X | X | X | X |
| **Safety: clinical6** |  |  |  | X |  |  |  | X | X |
| **Safety: bloods7** | X |  |  | X |  |  |  |  | X |

1. Run-in period requires 5 visits (including screening visit V1) over at least 2 weeks which, together with pre-treatment visit V6, comprise 6 sets of blood and urine samples prior to treatment

2. Each scheduled visit after entry has a 24 hr window with the exact time of sampling noted.

3. Standard history and physical examination, determining eligibility, providing written consent and pregnancy test

4. Venous serum and dried blood spots stored for later hormone analysis. Whole blood sample used for full blood count (including hemoglobin and reticulocytes).

5. Spot urine sample (at least 50 ml) for standard urine steroid profile plus CIR of testosterone and 5α reduced metabolites (DHT, epiandrosterone sulphate)

6. General well-being, emergent symptoms or adverse events

7. Clinical biochemistry (renal and liver function test)

**Background and relevance to anti-doping science**

The lure of fame and fortune through competitive success in elite sports will always tempt some athletes to cheat including by doping, the use of ergogenic drugs to improve performance. The two most potent ergogenic regimens remain androgen doping, notably for power sports, and hemoglobin doping, to enhance endurance. As a result, these two classes of ergogenic drugs remain the most detected forms of doping as judged by WADA statistics (1). Consequently, much anti-doping science research has focused on detection and deterrence of these dominant classes of banned hormonal drugs.

The potent ergogenic effects of increased hemoglobin, first proven in the early 1970s (2), led to use blood transfusion to increase circulating hemoglobin thereby enhancing oxygen transfer to muscle and other tissues in endurance sports. However, blood transfusion requires complex, sterile medical equipment, facilities and expertise to obtain, preserve and transfuse blood, a procedure involving risks of blood-borne infection and hemolysis. Effective detection of homologous blood transfusion (3) has largely eliminated the use of donated blood for doping although autologous blood donation remains difficult to detect. The cloning of erythropoietin (EPO) in the 1980s made available the more easily concealed technique of increasing hemoglobin by injection of EPO and its erythropoeitic stimulating agent (ESA) analogs (4). Hemoglobin doping has been countered by development of direct detection methods for EPO and ESAs in urine; however, these tests have a short window of detection. More effective testing with a wider window of detection has been developed using a Bayesian implementation of indirect hematological biomarkers as the hematological module of the Athlete’s Biological Passport (ABP). In turn, however, these effective indirect detection methods led to novel low dose (“microdosing”) regimens designed to circumvent detection of hemoglobin doping by EPO micro-dosing regimens which are reported to evade ABP testing (5-7).

Analogous developments have occurred in androgen doping. The use of synthetic androgens has become readily detectable even at long times after last dose through sensitive identification of distinctive xenobiotic chemical signatures and/or long-term metabolites. As a result, synthetic androgens now constitutes the largest category of adverse analytical findings in WADA-accredited anti-doping lab statistic (1). In reaction, attention has switched to using exogenous natural androgens, notably testosterone, which must be distinguished from its endogenous counterpart to confirm doping. Injectable or oral testosterone esters may be detected by the presence of a xenobiotic (testosterone ester) in the blood (8, 9); however, the use of non-esterified testosterone in transdermal testosterone gels, solution or patches remains challenging to detect. Conversely, the use of transdermal route of administration exposes testosterone to high levels of 5α reductase expressed in skin leading to a disproportionate pattern of 5α reduced metabolites of testosterone in circulating and urinary steroid profiles.

Exogenous non-esterified testosterone is detectable by urine tests, notably the urine T/E ratio backed-up with the carbon isotope ratio mass spectrometry (CIRMS) test; however, these tests have limitations. The urine T/E ratio, originally developed to use population-based reference ranges, has also been adapted with a Bayesian implementation of the urine T/E ratio into the steroidal ABP module. This requires a serially-adjusted, individual-specific thresholds whereby an individual’s result is compared with a pooling of all their own previous results. This creates a powerful individualized detection test based on narrower, individual-specific thresholds compared with population-based reference ranges. A limitation of the urine T/E ratio is the frequency of a genetic polymorphism in uridine diphospho-glucuronosyl transferase 2B17 (UGT2B17). This phase II metabolism enzyme glucuronidates most endogenous testosterone prior to its urinary excretion. Individuals with the homozygous deletor genotype have severely reduced urine testosterone excretion causing an order of magnitude lower T/E ratio (typically a mean 0.1 vs 1.0) compared with non-deletors, a feature which may mask exogenous testosterone use (10, 11). Furthermore, this deletor genotype/phenotype has a wide range of population prevalence, ranging between 5-70% among different ethnic groups (12-14). Nevertheless, the steroidal module of the ABP also adapts to the atypically low T/E ratio in deletors thereby enhancing the sensitivity of detection for testosterone doping even for individuals with the deletor UGT2B17 genetic polymorphism (15). Finally, the urine T/E ratio test depends in principle on the negative feedback of exogenous testosterone on epitestosterone secretion. Whereas this negative feedback system is operative in men with their single source of testosterone (i.e. 95% secreted from the testis), that negative feedback system that is largely inoperative in women whose circulating testosterone arises from three sources – adrenal, ovarian and extra-glandular conversion of precursors – none subject to potent negative feedback by testosterone as in men. This renders uncertain the applicability of the urine T/E ratio to detect testosterone doping in women.

The technically demanding, laborious and expensive CIRMS test identifies C13 isotope-depleted steroids of plant sterol origin (the conventional base sterol employed in commercial steroid manufacturing). The degree of C13 isotope depletion of a steroid (eg testosterone) purified from a urine sample can be compared with the mammalian (non-depleted) C13 isotope ratio allowing identification of an exogenous source (plant sterol vs mammalian synthesis) for the testosterone. However, this test may also be evaded by using the minority of commercial testosterone products manufactured from non-depleted plant sterol base materials whose C13 isotope ratios may overlap with endogenous human steroids making them difficult or impossible to detect by CIRMS testing (16-19).

Arising from these limitations, several novel analytical approaches to detect exogenous testosterone use have been reported. Among the most promising is the analysis of sulphate conjugates of testosterone metabolites which display more prolonged excretion profiles compared with the conventional glucuronidated conjugates. Hence sulphate conjugates of testosterone metabolites constitute a category of longer-term metabolites suitably for monitoring to provide longer detection windows. Notably, studies the CIR of epiandrosterone sulphate has been reported to display prolonged detectability as a marker of exogenous testosterone use (20).

Other recently proposed approaches to detect exogenous testosterone doping are less promising as practical antidoping strategies. For example, while salivary testing has the advantage of being minimally invasive sampling as a potential test to detect exogenous testosterone (21-23), steroid concentrations in saliva are influenced by numerous physiological factors including age, sex, stress, hormones (menstrual cycle, hormonal contraceptives) and collection time of day. Individuals (notably athlete during events) may have difficulty producing sufficient saliva but using various stimuli to salivary secretion influences steroid concentrations. Ultimately however, saliva is an unsatisfactory biological fluid matrix due to the risk of contamination by blood which has circulating levels 50 times higher than in saliva which in practice leads unpredictably to “outlier” results (23). Hence, even microscopic blood contamination (eg due to poor dental hygiene, eating hard food, tooth brushing etc) could lead to false positives and indefensible medico-legal challenge to the validity of saliva testing. Another proposed approach has been measurements of circulating microRNA-122 following exogenous testosterone administration (24); however, this could only be a non-specific, generic biomarker of androgen action and the findings have not been replicated (25).

Studies over more than 2 decades have examined the use of serum steroids and related analytes (LH, SHBG) to detect exogenous testosterone administration (22, 26, 27). Although urinary steroids display a more stable integrated pattern than serum steroids (28), within the window of detection serum measurements may be more sensitive. Despite the anecdotal evidence that women also abuse androgens including testosterone, so far to the best of our knowledge, there are no systematic anti-doping studies of exogenous testosterone administration in women to develop and evaluate detection tests. Few antidoping studies have investigated transdermal administration of testosterone (20, 21, 25, 29, 30) with those studies restricted to one (21, 25), two (29), three (30) or seven (20) doses in men but none in women. From these studies important suggestions for better long-term metabolites for monitoring to detect testosterone microdosing have included disproportionate increases in serum DHT and reticulocytes (25) and the CIR of epiandrosterone sulphate (20). Overall, it remains to be evaluated how well the serum or urine steroid profile in conjunction with the hematological profile may be effective for identifying exogenous testosterone administration in women.

Although there is theoretical uncertainty about whether the urine T/E ratio is effective for detection of testosterone doping in women, there is conversely a strong likelihood that the known erythropoeitic effects of exogenous androgens would be especially prominent in women thereby forming the basis of an additional detection method for testosterone doping in women. The sex difference between men and women in circulating hemoglobin of 12% (31) is attributable to the differences in circulating testosterone(32). Administration of testosterone to healthy men increases blood hemoglobin and circulating erythropoietin within 4 days while cessation of testosterone by long-term androgen abusers led to reduced hemoglobin (33). Hence, the use of exogenous androgens like testosterone would reverse this sex difference providing an ergogenic advantage in women (34) but also providing an opportunity for enhanced detection of exogenous testosterone doping through integrating the steroidal and hematological modules of the ABP which are usually considered independently.

This proposal aims to employ serum steroid (and LH) profiling as well as the hematological module of the ABP (off-score) in conjunction with the conventional urine steroid (and LH) profile to determine the optimal combination for sensitive detection of testosterone micro-dosing in women.

**Hypothesis and specific objectives**

This study aims to determine the effectiveness (sensitivity and specificity) of the serum and urine steroid (and LH) profiles plus the hematological profile individually and in optimized combination to detect testosterone micro-dosing in women. The analysis will proceed in several stages.

In the first stage we will compare conventional urine steroid profiling with (a) serum steroid (plus LH and FSH) profile, (b) the CIR of testosterone and certain 5α reduced metabolites (DHT and epiandrosterone sulphate) both as absolute values and as δ-δ values compared with a reference steroid and (c) full blood count (including hemoglobin and reticulocyte count). In addition, we will use the ADAMS Athlete Biological Passport software to review steroid and hematological profiles.

Subsequently we will combine all the above variables into a single data matrix to be analyzed by principal component or linear discriminant analysis to identify the best, unbiased combination of variables (both in absolute terms and in differences from baseline) that differentiate effectively between baseline pre-treatment and on testosterone treatment.

**Experimental design, methods, and data analysis (please include a timeline of major milestones and deliverables) \***

*Study design*: Open label study with blood and urine sampling during (a) run-in period over at least 2 weeks to establish individual baselines (6 pre-treatment paired blood and urine samples), (b) 7 days of daily testosterone treatment (12.5 mg topical testosterone daily) with (c) 14 days of post-treatment run-off (5 post-treatment blood and urine samples over 2 weeks).

*Study population:* Volunteers will be recruited by local advertising via electronic, print and social media. They must be eligible according to entry criteria and provide signed informed consent acknowledging their willingness to participate fully in the study. Participants will be reimbursed for their time and attendance at the clinic. They may participate in the study more than once after an interval of at least a month following completion of the study.

*Sample size:* The sample size of 12 women is based on the local prevalence of the UGT2B17 deletor phenotype (30%) so that we expect to get 4 with the deletor phenotype. The repeated measures design for a single group with six samples before and six samples after treatment provides 92% power (two-sides α 0.05) to detect within person changes of at least 0.45 standard deviation for analytes mean value after compared with before for 12 participants, allowing for a 10% drop-out rate. A further refined analysis assuming the major changes will be in the first 3 post-treatment samples provides 92% power to detect 0.4 times the standard deviation for each analyte.

***Entry criteria:***

Inclusion criteria:

* Healthy women over 18 years of age
* Willing to provide written, informed consent and to participate fully in all study requirements

Exclusion criteria:

* Pregnant, breast feeding or likely to be seeking fertility within next 6 months
* Not using hormonal drugs other than hormonal contraception (eg estrogen replacement)
* Athletes subject to regular urine drug testing
* Contraindications to testosterone administration (breast cancer or polycythemia)
* Major or chronic medical disorders requiring regular prescribed medication;
* Severe or extensive skin disease which interferes with transdermal application of testosterone gel
* Regular medications that interferes with testosterone metabolism
* History of androgen or other drug abuse within last year
* Major psychiatric disease or psychological condition that limits understanding and compliance with study requirements in the opinion of the investigator

Precautions:

* Working in an occupation (other than athletics) that requires urine drug testing

*Treatments:* Testosterone treatment will be provided by Testogel (Besins Healthcare), a 1% (w/v) transdermal testosterone gel in a metered dose pump pack bottle (ARTG 227563). Each actuation of the pump provides 1.25 gram of the 1% gel. The daily dose will be 1 pump actuation/day thereby providing a daily topical dose of 12.5 mg testosterone.

***Study endpoints:***

The primary endpoints will be the steroid and urine steroid profiles measured by MS-based methods and the hematological profile (including but not restricted to the OFF-score). Blood samples will be taken for hematological analysis on the same day. Serum and urine samples will be frozen at -20 C until ready for batch assays.

The serum steroids to be measured will be testosterone (T), dihydrotestosterone (DHT), estradiol, estrone, DHEA, 3α 5α androstanediol (3α diol) and 3β 5α androstanediol (3β diol)). Dried blood spots will also be analyzed for testosterone.

The urine steroid profile will comprise measurement of T, epitestosterone (EpiT), androsterone (A), etiocholanolone (Etio), dehydroepiandrosterone (DHEA), DHT, 5α 3α androstanediol, 5β 3α androstanediol together with the following ratios of T/EpiT (T/E), A/Etio (A/E) and 5α 3α androstanediol/5β 3α androstanediol (5α/5β).

Urine CIRMS testing of testosterone and certain 5α reduced metabolites (DHT, androsterone, etiocholanolone, epiandrosterone sulphate)

The hematological variables to be measured include hemoglobin, reticulocytes (number and %) and other standard erythrocyte metrics (hematocrit, erythrocyte count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) as provided by the hematology autoanalyzer.

***Sample processing and management:***

**Methods**

Serum steroids (testosterone (T), dihydrotestosterone (DHT), estradiol, estrone, DHEA, 3α 5α androstanediol (3α diol) and 3β 5α androstanediol (3β diol)) will be measured by liquid chromatography-mass spectrometry (LC-MS) using published methods as originally described (35), modified to include ultrapressure liquid chromatography and different liquid:liquid extraction (36) and with extensive longitudinal quality control (37) reported previously. Serum LH and FSH are measured by human gonadotropin immunoassays (Roche).

The LC–MS analysis is based on reversed-phase chromatographic separation of the injected sample using UPLC followed by gradient elution and detection of the targeted steroids using triple quadrupole mass spectrometric analysis. The liquid chromatography system was a Shimadzu Nexera UHPLC with a Restek Raptor biphenyl column (100 cm × 2.1 mm, 2.7 µm) with Raptor biphenyl guard cartridges. The solvents used are; A Milli-Q water, B methanol, and C toluene (dopant). The injected sample (50 µL) is initially washed with 10% B followed by a gradient comprising: 0.11-2.0 min 25-50%B, 2.00-10.0 min 50-60%B, 10.01-13.5min 65-69%B, 13.5-18.0 min 69-75%B, 18.01-20.0 min 85-95%B, then 100%B until 20.5 mins and then back to 10% for 1 min. The flow rate is 0.7 mL/min for a total run time of 21.5 mins with column temperature maintained at 40 °C and samples at 4 °C.

The API-5000 triple-quadruple mass spectrometer (Applied Biosystems /MDS SCIEX, Ontario, Canada) is equipped with an atmospheric pressure photoionization (APPI) source employing a 10 eV krypton discharge lamp with dopant (toluene) delivery set to 75 µL/min. The APPI probe temperature is 500 °C and the ion spray voltage set to 750 V in positive and −750 V in negative mode. Steroids are quantified by multiple reaction monitoring (MRM) using settings for the various transitions optimized by infusing pure steroid into the mass spectrometer. Unit mass resolution was used in both mass-resolving quadruples Q1 and Q3. Data acquisition and processing was performed with Analyst 1.6.2 (AB SCIEX). Peak area ratios of analyte and IS quantifier transitions were calculated as a function of analyte concentration. Calibration curves and lower limits of quantification (LOQ) were defined according to the FDA guidance. Dried blood spots will be analyzed by methods previously established (38).

The urine steroid profile including T/E ratio and LH (by immunoassay) will be measured by standard WADA GC-MS methods in a WADA-accredited anti-doping laboratory maintains (39) ongoing accreditation by participation in the WADA’s External Quality Assessment Scheme (EQAS)(40). Briefly, after extraction and deconjugation, urine T, epitestosterone (EpiT), androsterone (A), etiocholanolone (Etio), dehydroepiandrosterone (DHEA), DHT, 5α 3α androstanediol, 5β 3α androstanediol, T/EpiT (T/E), A/Etio (A/E) and 5α 3α androstanediol/5β 3α androstanediol (5α/5β) ratios are measured by standard GC-MS methods using routine GC-MS steroid profiling (41) according to WADA standard requirements for measurement of endogenous androgens (39). Carbon isotope ratio (CIR) measurements will be performed by previously described method (42) for steroids including testosterone, androsterone, etiocholanolone, 5α,3α androstanediol, 5β, 3α androstanediol and epiandrosterone sulphate (20). Urine LH is measured by a specific human LH immunoassay (40).

The uridine 5'-diphospho-glucuronosyltransferase (UGT) 2B17 genotyping will be performed by polymerase chain reaction classifying genotypes into deletor and non-deletor according to homozygous wild-type (CC), heterozygous (CJ) and homozygous deletor (JJ) as reported previously (43).

A hematological profile comprising full blood count (including haemoglobin, reticulocyte counts and erythrocyte metrics) is measured by a regularly calibrated, automated hematology analyser.

***Data analysis***

The serial results of the serum and urine steroids, serum LH and FSH and urine LH, hematological profile will be analyzed using linear mixed model linear analysis for repeated measures including the run-in, treatment and run-off periods with dose as the main effect and age and body weight (or BMI) as covariates. Linear contrasts will be used to highlight the treatment effects by comparing the means from from run-in and run-off periods.

For analytes where there is a significant difference from baseline receiver-operated curve (ROC) analysis will be used to define the optimal cutpoint for individual (absolute and difference from baseline) and combined parameters to detect testosterone administration as well as the window of detection during run-off.

***Safety***

This short-term study poses minimal risks to the healthy participants. Warnings will be provided in the Information Sheet regarding any possible androgenic symptoms resulting from testosterone treatment.

The study will have a local, independent Data Safety and Monitoring Board chaired by Professor Andrew McLachlan, Dean of Pharmacy, University of Sydney, an experienced clinical pharmacologist, anti-doping expert and former Chair of Human Ethics Committee of Sydney Local Health District.

In women, any exogenous testosterone is supra-physiological, off-label use and needs to be accompanied by warning regarding possibility of virilization (acne, hair growth, voice change). The virilizing effects of testosterone in females, best studied in transgender men (F2M transgender individuals) who take full male testosterone doses, take months to become manifest (44). Nevertheless, the investigators will warn and review participants for any emergent signs of virilization and, if reported, discontinue the treatment. In that setting of short-term use, any recently emergent side-effects, should they occur, would be expected to be quickly and fully reversible. The information sheet will warn participants of the potential to transiently impair their fertility and to be detected in occupational urine drug screens.

The testosterone doses used in this short-term study are comparable with doses used safely in large scale, phase III clinical trials of transdermal testosterone for women with sexual dysfunction. Many previous studies in women have used transdermal testosterone in either gel, cream or patch form. In a meta-analysis of 7 studies of 3035 women (1350 on testosterone) using a testosterone patch that notionally delivered 300 µg testosterone daily for 6 months, there was no significant overall effect on total or severe androgenic effects, withdrawal or follow-up rate from the study although in subsets of studies there was a significant increase in prevalence of acne or hirsutism but not in facial hair, alopecia or voice deepening (45). Those findings were confirmed by a reported increase in prevalence of acne and hirsutism in another meta-analysis that included 35 randomized controlled trials of testosterone therapy in 5053 women treated with any form of testosterone or dose for a median of 6 months (range 1.5 to 24 months) (46). Higher doses of transdermal testosterone patch (450 µg/day) did not increase rate of androgenic side-effects (47). As the bioavailability of testosterone from a transdermal patch is estimated at 3% in healthy women (48), a patch notionally delivering 300 µg/day would be equivalent of a testosterone gel applying of 10 mg of testosterone to the skin daily. Other studies in healthy women have used a daily transdermal application of 10 mg testosterone daily with a similar safety profile (49-52). The doses used in this 7 day study are comparable but used for a much shorter period of time.

***Adverse Effects***

All emergent or adverse events will be recorded. Any serious adverse effects notified to the SLHD Human Ethics Committee.

***Ethics and governance***

The study will be approved by the SLHD Human Ethics Committee (Concord Hospital) and notified to the Therapeutic Goods Administration (TGA) under the Clinical Trial Notification (CTN) scheme for use of testosterone outside its approved indications.

**Facilities and equipment \***

The clinical studies will be performed in the Andrology Department, Concord Hospital which is fully equipped for such studies. Numerous clinical studies, including anti-doping investigations (43, 53), have been conducted and reported by the same clinic and laboratory staff with approval from the local Human Ethics Committee.

The ANZAC Research Institute has all the necessary facilities to conduct and equipment required for this study other than the urine steroid profiling which will be undertaken by a WADA-accredited anti-doping laboratory. The serum steroid profiling will performed within the ANZAC Research Institute’s Andrology laboratory on a Shimadzu Nexera UHPLC liquid chromatography system with a Restek Raptor biphenyl column coupled to an API-5000 triple-quadruple mass spectrometer. The serum LH and FSH immunoassays will be performed by the Concord Hospital Diagnostic Pathology Unit located at the same teaching hospital campus.

The Sport Medicine Research and Testing Laboratory (SMRTL) is accredited by the WADA and ISO 17025 in 2006. WADA accreditation assures that testing methods meet the highest and latest standards, new tests are developed and applied, periodic on-site audits verify compliance with standards and research and development is routinely undertaken. The American Association of Laboratory Accreditation (A2LA) verifies that SMRTL meets the International Organization for Standardization (ISO) requirements for its ISO 17025 standards. This accreditation assures testing methods and processes are fully documented, scientifically validated, publicly available for review and periodically updated. SMRTL currently tests over 50,000 specimens annually for steroid profiles and ~5000 for IRMS analysis. Further, SMRTL has completed several testosterone research studies.

**References cited \***

1. **WADA** 2015 Anti-Doping Testing Figures - Laboratory Report: pages <https://www.wada-ama.org/sites/default/files/resources/files/2015_wada_anti-doping_testing_figures_report_2010.pdf>. Available at

2. **Ekblom B, Goldbarg AN, Gullbring B** 1972 Response to exercise after blood loss and reinfusion. J Appl Physiol 33:175-180

3. **Nelson M, Popp H, Sharpe K, Ashenden M** 2003 Proof of homologous blood transfusion through quantification of blood group antigens. Haematologica 88:1284-1295

4. **Elliott S** 2008 Erythropoiesis-stimulating agents and other methods to enhance oxygen transport. Br J Pharmacol 154:529-541

5. **Ashenden M, Gough CE, Garnham A, Gore CJ, Sharpe K** 2011 Current markers of the Athlete Blood Passport do not flag microdose EPO doping. Eur J Appl Physiol 111:2307-2314

6. **Dehnes Y, Shalina A, Myrvold L** 2013 Detection of recombinant EPO in blood and urine samples with EPO WGA MAIIA, IEF and SAR-PAGE after microdose injections. Drug Test Anal 5:861-869

7. **Clark B, Woolford SM, Eastwood A, Sharpe K, Barnes PG, Gore CJ** 2017 Temporal changes in physiology and haematology in response to high- and micro-doses of recombinant human erythropoietin. Drug Test Anal 9:1561-1571

8. **Tretzel L, Thomas A, Geyer H, Gmeiner G, Forsdahl G, Pop V, Schanzer W, Thevis M** 2014 Use of dried blood spots in doping control analysis of anabolic steroid esters. J Pharm Biomed Anal 96:21-30

9. **Forsdahl G, Erceg D, Geisendorfer T, Turkalj M, Plavec D, Thevis M, Tretzel L, Gmeiner G** 2015 Detection of testosterone esters in blood. Drug Test Anal 7:983-989

10. **Schulze JJ, Lundmark J, Garle M, Skilving I, Ekstrom L, Rane A** 2008 Doping test results dependent on genotype of uridine diphospho-glucuronosyl transferase 2B17, the major enzyme for testosterone glucuronidation. J Clin Endocrinol Metab 93:2500-2506

11. **Schulze JJ, Lundmark J, Garle M, Ekstrom L, Sottas PE, Rane A** 2009 Substantial advantage of a combined Bayesian and genotyping approach in testosterone doping tests. Steroids 74:365-368

12. **Xue Y, Sun D, Daly A, Yang F, Zhou X, Zhao M, Huang N, Zerjal T, Lee C, Carter NP, Hurles ME, Tyler-Smith C** 2008 Adaptive evolution of UGT2B17 copy-number variation. Am J Hum Genet 83:337-346

13. **Martin-Escudero P, Munoz-Guerra J, Del Prado N, Galindo Canales M, Fuentes Ferrer M, Vargas S, Soldevilla AB, Serrano-Garde E, Miguel-Tobal F, Maestro de Las Casas M, Fernandez-Perez C** 2015 Impact of UGT2B17 gene deletion on the steroid profile of an athlete. Physiol Rep 3

14. **Okano M, Ueda T, Nishitani Y, Kano H, Ikekita A, Kageyama S** 2013 UDP-glucuronosyltransferase 2B17 genotyping in Japanese athletes and evaluation of the current sports drug testing for detecting testosterone misuse. Drug Test Anal 5:166-181

15. **Strahm E, Mullen JE, Garevik N, Ericsson M, Schulze JJ, Rane A, Ekstrom L** 2015 Dose-dependent testosterone sensitivity of the steroidal passport and GC-C-IRMS analysis in relation to the UGT2B17 deletion polymorphism. Drug Test Anal 7:1063-1070

16. **Cawley A, Collins M, Kazlauskas R, Handelsman DJ, Heywood R, Longworth M, Arenas-Queralt A** 2010 Stable isotope ratio profiling of testosterone preparations. Drug Test Anal 2:557-567

17. **Forsdahl G, Ostreicher C, Koller M, Gmeiner G** 2011 Carbon isotope ratio determination and investigation of seized testosterone preparations. Drug Test Anal 3:814-819

18. **Hullstein I, Sagredo C, Hemmersbach P** 2014 Carbon isotope ratios of nandrolone, boldenone, and testosterone preparations seized in Norway compared to those of endogenously produced steroids in a Nordic reference population. Drug Test Anal 6:1163-1169

19. **Brooker L, Cawley A, Drury J, Edey C, Hasick N, Goebel C** 2014 Stable carbon isotope ratio profiling of illicit testosterone preparations--domestic and international seizures. Drug Test Anal 6:996-1001

20. **Piper T, Putz M, Schanzer W, Pop V, McLeod MD, Uduwela DR, Stevenson BJ, Thevis M** 2017 Epiandrosterone sulfate prolongs the detectability of testosterone, 4-androstenedione, and dihydrotestosterone misuse by means of carbon isotope ratio mass spectrometry. Drug Test Anal 9:1695-1703

21. **Thieme D, Rautenberg C, Grosse J, Schoenfelder M** 2013 Significant increase of salivary testosterone levels after single therapeutic transdermal administration of testosterone: suitability as a potential screening parameter in doping control. Drug Test Anal 5:819-825

22. **Schonfelder M, Hofmann H, Schulz T, Engl T, Kemper D, Mayr B, Rautenberg C, Oberhoffer R, Thieme D** 2016 Potential detection of low-dose transdermal testosterone administration in blood, urine, and saliva. Drug Test Anal 8:1186-1196

23. **Polet M, De Wilde L, Van Renterghem P, Van Gansbeke W, Van Eenoo P** 2018 Potential of saliva steroid profiling for the detection of endogenous steroid abuse: Reference thresholds for oral fluid steroid concentrations and ratios. Anal Chim Acta 999:1-12

24. **Salamin O, Jaggi L, Baume N, Robinson N, Saugy M, Leuenberger N** 2016 Circulating microRNA-122 as Potential Biomarker for Detection of Testosterone Abuse. PLoS One 11:e0155248

25. **Mullen J, Borjesson A, Hopcraft O, Schulze JJ, Ericsson M, Rane A, Lehtihet M, Ekstrom L** 2017 Sensitivity of doping biomarkers after administration of a single dose testosterone gel. Drug Test Anal

26. **Carlstrom K, Palonek E, Garle M, Oftebro H, Stanghelle J, Bjorkhem I** 1992 Detection of testosterone administration by increased ratio between serum concentrations of testosterone and 17 alpha-hydroxyprogesterone. Clin Chem 38:1779-1784

27. **Ponzetto F, Mehl F, Boccard J, Baume N, Rudaz S, Saugy M, Nicoli R** 2016 Longitudinal monitoring of endogenous steroids in human serum by UHPLC-MS/MS as a tool to detect testosterone abuse in sports. Anal Bioanal Chem 408:705-719

28. **Savkovic S, Lim S, Jayadev V, Conway A, Turner L, Curtis D, Goebel C, Handelsman DJ** 2018 Urine and Serum Sex Steroid Profile in Testosterone-Treated Transgender and Hypogonadal and Healthy Control Men. J Clin Endocrinol Metab 103:2277-2283

29. **Badoud F, Boccard J, Schweizer C, Pralong F, Saugy M, Baume N** 2013 Profiling of steroid metabolites after transdermal and oral administration of testosterone by ultra-high pressure liquid chromatography coupled to quadrupole time-of-flight mass spectrometry. J Steroid Biochem Mol Biol 138:222-235

30. **Kotronoulas A, Gomez-Gomez A, Fabregat A, Segura J, Yang S, Xing Y, Moutian W, Marcos J, Joglar J, Ventura R, Pozo OJ** 2018 Evaluation of markers out of the steroid profile for the screening of testosterone misuse. Part I: Transdermal administration. Drug Test Anal 10:821-831

31. **Murphy WG** 2014 The sex difference in haemoglobin levels in adults - mechanisms, causes, and consequences. Blood Rev 28:41-47

32. **Karunasena N, Han TS, Mallappa A, Elman M, Merke DP, Ross RJ, Daniel E** 2017 Androgens correlate with increased erythropoiesis in women with congenital adrenal hyperplasia. Clin Endocrinol (Oxf) 86:19-25

33. **Mullen JE, Schulze JJ, Rane A** 2014 Perturbation of the Hematopoietic Profile by Anabolic Androgenic Steroids. Journal of Hormones 2014:1-7

34. **Handelsman DJ, Hirschberg AL, Bermon S** 2018 Circulating Testosterone as the Hormonal Basis of Sex Differences in Athletic Performance. Endocr Rev

35. **Harwood DT, Handelsman DJ** 2009 Development and validation of a sensitive liquid chromatography-tandem mass spectrometry assay to simultaneously measure androgens and estrogens in serum without derivatization. Clin Chim Acta 409:78-84

36. **Keski-Rahkonen P, Desai R, Jimenez M, Harwood DT, Handelsman DJ** 2015 Measurement of Estradiol in Human Serum by LC-MS/MS Using a Novel Estrogen-Specific Derivatization Reagent. Anal Chem 87:7180-7186

37. **Hsu B, Cumming RG, Hirani V, Blyth FM, Naganathan V, Le Couteur DG, Seibel MJ, Waite LM, Handelsman DJ** 2016 Temporal Trend in Androgen Status and Androgen-Sensitive Outcomes in Older Men. J Clin Endocrinol Metab 101:1836-1846

38. **Singh GK, Turner L, Desai R, Jimenez M, Handelsman DJ** 2014 Pharmacokinetic-pharmacodynamic study of subcutaneous injection of depot nandrolone decanoate using dried blood spots sampling coupled with ultrapressure liquid chromatography tandem mass spectrometry assays. J Clin Endocrinol Metab 99:2592-2598

39. **WADA** 2016 Technical document for endogenous anabolic androgenic steroids (TD2016EAAS): pages Available at <https://www.wada-ama.org/en/resources/science-medicine/td2016eaas>

40. **WADA** 2016 International Standard for Laboratories (ISL): pages Available at <https://www.wada-ama.org/en/resources/laboratories/international-standard-for-laboratories-isl>

41. **Goebel C, Howe CJ, Ho KK, Nelson A, Kazlauskas R, Trout GJ** 2009 Screening for testosterone abuse in male athletes using the measurement of urinary LH, a revision of the paradigm. Drug Test Anal 1:511-517

42. **Miller GD, Nair V, Morrison MS, Summers M, Willick SE, Eichner D** 2016 Intranasal delivery of Natesto(R) testosterone gel and its effects on doping markers. Drug Test Anal 8:1197-1203

43. **Handelsman DJ, Idan A, Grainger J, Goebel C, Turner L, Conway AJ** 2014 Detection and effects on serum and urine steroid and LH of repeated GnRH analog (leuprolide) stimulation. J Steroid Biochem Mol Biol 141:113-120

44. **Nakamura A, Watanabe M, Sugimoto M, Sako T, Mahmood S, Kaku H, Nasu Y, Ishii K, Nagai A, Kumon H** 2013 Dose-response analysis of testosterone replacement therapy in patients with female to male gender identity disorder. Endocr J 60:275-281

45. **Achilli C, Pundir J, Ramanathan P, Sabatini L, Hamoda H, Panay N** 2017 Efficacy and safety of transdermal testosterone in postmenopausal women with hypoactive sexual desire disorder: a systematic review and meta-analysis. Fertil Steril 107:475-482 e415

46. **Elraiyah T, Sonbol MB, Wang Z, Khairalseed T, Asi N, Undavalli C, Nabhan M, Firwana B, Altayar O, Prokop L, Montori VM, Murad MH** 2014 The Benefits and Harms of Systemic Testosterone Therapy in Postmenopausal Women With Normal Adrenal Function: A Systematic Review and Meta-analysis. J Clin Endocrinol Metab 99:3543-3550

47. **Braunstein GD, Sundwall DA, Katz M, Shifren JL, Buster JE, Simon JA, Bachman G, Aguirre OA, Lucas JD, Rodenberg C, Buch A, Watts NB** 2005 Safety and efficacy of a testosterone patch for the treatment of hypoactive sexual desire disorder in surgically menopausal women: a randomized, placebo-controlled trial. Arch Intern Med 165:1582-1589

48. **Javanbakht M, Singh AB, Mazer NA, Beall G, Sinha-Hikim I, Shen R, Bhasin S** 2000 Pharmacokinetics of a novel testosterone matrix transdermal system in healthy, premenopausal women and women infected with the human immunodeficiency virus. J Clin Endocrinol Metab 85:2395-2401

49. **El-Hage G, Eden JA, Manga RZ** 2007 A double-blind, randomized, placebo-controlled trial of the effect of testosterone cream on the sexual motivation of menopausal hysterectomized women with hypoactive sexual desire disorder. Climacteric 10:335-343

50. **Barton DL, Wender DB, Sloan JA, Dalton RJ, Balcueva EP, Atherton PJ, Bernath AM, Jr., DeKrey WL, Larson T, Bearden JD, 3rd, Carpenter PC, Loprinzi CL** 2007 Randomized controlled trial to evaluate transdermal testosterone in female cancer survivors with decreased libido; North Central Cancer Treatment Group protocol N02C3. J Natl Cancer Inst 99:672-679

51. **Nathorst-Boos J, Floter A, Jarkander-Rolff M, Carlstrom K, Schoultz B** 2006 Treatment with percutanous testosterone gel in postmenopausal women with decreased libido--effects on sexuality and psychological general well-being. Maturitas 53:11-18

52. **Goldstat R, Briganti E, Tran J, Wolfe R, Davis SR** 2003 Transdermal testosterone therapy improves well-being, mood, and sexual function in premenopausal women. Menopause 10:390-398

53. **Handelsman DJ, Goebel C, Idan A, Jimenez M, Trout G, Kazlauskas R** 2009 Effects of recombinant human LH and hCG on serum and urine LH and androgens in men. Clinical Endocrinology 71:417-428