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Steroid profiling among National Football League players

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Abstract

The detection of the abuse of endogenously occurring steroids continues to be a challenge for laboratories and anti-doping organizations alike. The use of steroid profiling information has proven to be a valuable tool in this endeavour. Evaluating the concentrations and ratios of various naturally occurring steroids, their precursors, and metabolites provides a wealth of information. Population based reference studies have been used by WADA to establish critical limits for various steroid concentrations and ratios. In addition, individual profiling is increasingly being used as a more sensitive and selective tool for monitoring single athletes, as intra-individual variability is generally much smaller that population based ranges. We have proposed to make use of retrospectively collected steroid data from National Football League (NFL) players to develop an adaptive model that will be used to evaluate longitudinal steroid profiles of individual athletes. The database consists of over 17,000 samples from nearly 3,100 individual players. In addition, there are more than 600 players with 10 or more tests, giving us a large group suitable for individual longitudinal profiling. Evaluation of the population distributions reveals that, while there are many similarities with previous studies, significant differences exist between this population of athletes and other populations of elite athletes. For example, the median T/E ratio of 1.06 is nearly identical to the value of 1.09 reported by Catlin et al.(Catlin et al., 1997) in a previous study of NFL players, but differs substantially from the value of 1.39 reported recently for a population consisting of almost exclusively Caucasians.(Van Renterghem et al., 2010) These differences reinforce the need to consider the makeup of a particular population when applying reference ranges from groups that may not be entirely representative of the target population. Examples from individual players are also presented, and demonstrate the usefulness of evaluating multiple parameters in the steroid profile.

Introduction

Alterations in the urinary excretion patterns of endogenous steroids have long been employed to facilitate the detection of doping with banned substances. The most common approach has

been to measure the testosterone (T) to epitestosterone (E) ratio (T/E). This approach was adopted by the International Olympic Committee (IOC) in 1982 (Donike et al., 1983) using a population-based ratio of 6.0 as a threshold. In 2005, the World Anti-Doping Agency (WADA) lowered the reporting threshold to 4.0 (WADA, 2004). Population thresholds for other steroids such as androsterone (Andro), etiocholanolone (Etio), T, E and DHEA have also been established. However, when biomarker behavior, such as the T/E ratio or other endogenous steroid concentration is heterogeneous across individuals, a better approach is to use a longitudinal algorithm to tailor the threshold to individual history. This approach is appropriate for endogenous steroids, where the intra-individual variability is low (Mareck-Engelke et al., 1992). Donike et al. (Donike et al., 1993) proposed the use of subject-based reference ranges for T/E profiling rather than population-based reference ranges as being more sensitive to intra-individual variations. Recently, Sottas et al. have validated a Bayesian approach for the longitudinal monitoring of the T/E ratio (Sottas et al., 2007).

The urinary steroid profile is composed of concentrations and ratios of endogenously produced steroid hormones, their precursors, and metabolites including commonly T, E, Andro, Etio, 5α androstane-3α, 17β-diol (A-diol), 5β-androstane-3α,17β-diol (B-diol), and dehydroepiandrosterone (DHEA). Additional precursors and metabolites may also be considered as potential analytes to support the detection of surreptitious administration of endogenous steroids as well as bacterial activity (Shackleton et al., 1997). Many synthetic, or exogenous steroids can have significant impacts on the endogenous steroid profile as well (Mareck et al., 2008).

Numerous studies have investigated steroid parameters among different populations of athletes. (Ayotte et al., 1996; Baenzinger and Bowers, 1994; Catlin et al., 1997; Donike et al., 1992; Van Renterghem et al., 2010). Some of them analyzed just T/E ratios (Baenzinger and Bowers, 1994; Catlin et al., 1997) while others looked at relatively small populations of specific athletes (Donike et al., 1992) or ethnically homogenous populations (Van Renterghem et al., 2010). With a goal in mind of developing an adaptive model for individual steroid profiling of NFL players, we wanted to establish population reference ranges directly applicable to the ethnically diverse and physically unique population of the NFL.

We have compiled a database of 17,788 samples from 3,108 National Football League players, analyzed at our laboratory from 2006-2008. We have analyzed the endogenous

steroid parameters of this population. This was done by sample and by player, and resulted in descriptive statistics for all of the concentrations and steroid ratio parameters. Finally, we have begun evaluating adaptive models in order to determine the most appropriate one to use for our studies.

Methods

Quantitative steroid screening: Internal standard was added to 3 ml urine aliquots, which were then buffered with 1 ml of 0.8 M potassium phosphate buffer (pH 7.0). The samples were hydrolyzed with 25 µl of β-glucuronidase, and buffered for extraction with 0.75 ml of a 20% solution (w/v) of $K_2CO_3/KHCO_3$. 6 ml MTBE was added, and the samples were shaken for 10 min. followed by a 10 min. centrifugation. The ether layer was transferred to a new 13 x 100 mm silanized glass tube and evaporated under air at 40°C. Samples were derivatized with MSTFA:NH_4I:ethanethiol (1000 mcl:2 mg:10 mcl) at 75°C for 30 min. 3 µl was injected on to an Agilent GC/MSD and analyzed by selected ion monitoring (SIM).

Data analysis

Because these data were not controlled (i.e., we do not have knowledge of positive vs. negative samples), we needed to "clean up" or refine this database. This involved several steps. 1) We removed all known positive samples from the database, as these samples would clearly bias our reference population. 2) We removed 221 samples that had too many missing values to be of use. 3) To control for different hydration states, all concentration data were normalized to a specific gravity of 1.02 using the following equation:

$$(1.02-1)/(\text{S.G. [sample]} -1)$$

4) We removed obviously extreme concentration values from the database (e.g., Andro or Etio >25,000 ng/ml; T, E, or DHEA > 300 ng/ml), and 5) We removed all statistically determined outliers. Outliers were determined using a 1000 iteration bootstrap procedure on the upper quartile of the data. The double of the 99th percentile was determined and used as an outlier threshold.

Most statistical calculations were carried out using STATA. Microsoft Excel was used to calculate population reference limits. Summary statistics were calculated as follows: For individual tests, the individual variables in the refined database were used to calculate summary statistics such as mean, median, S.D., minimum and maximum. For player means, the mean of each player in the refined database was calculated. Those means were then used to calculate summary statistics by player.

Results

The results from our outlier determination are shown in Table 1. Note that in each case, the outlier factor is well above WADA's population based threshold limit of that particular parameter. Comparing the prior mean and standard deviation (S.D.) with the new mean and S.D. (after outlier removal) demonstrates that the removal of outliers has a negligible impact on our steroid variables and is a testament to the power of having such a large database. Even if there are some undetected positives (false negatives) remaining, they should have an insignificant impact on our population statistics.

Table 1 – Summary of outlier determination

Parameter	Mean	Std. Dev.	Outlier Factor	Outliers Removed	New Mean	New Std. Dev
Corrected T/E	1.289	1.041	12.360	1	1.286	1.006
Testosterone	35.4	20.5	264.0	1	35.3	20.0
Epitestosterone	38.3	24.9	322.0	1	38.3	24.3
Androsterone	2725	1378	18730	0	2725	1378
Etiocholanolone	1930	997	14094	0	1930.0	997
DHEA	35.5	21.5	302.0	0	35.5	21.5
Andro/Etio	1.57	0.87	9.96	8	1.56	0.72
Andro/Test	100.4	65.9	668.3	1	100.3	65.4

Our final database consists of more than 17,000 tests from nearly 3100 athletes. In addition, this database contains 637 athletes with 10 or more samples. Table 2 lists the summary statistics for all of the remaining samples in the database. The numbers of tests for T and the A/T ratio are lower than the others because we were not quantitating below 10 ng/ml at the time these samples were analyzed. WADA's reporting thresholds align quite well with either the 99^{th} or 99.9^{th} percentile of each parameter, and most of the means and/or medians are in close agreement with previous studies of similar magnitude (Donike et al., 1993; Geyer et al.,

1997; Van Renterghem et al., 2010). However, the values for T/E ratio do show some significant differences from certain studies of large populations (Geyer et al., 1997; Van Renterghem et al., 2010). One key variable, however, is that both of these studies were done with primarily northern European men. Van Renterghem et al. in particular, estimated that 99.5% of their population consisted of Caucasian males. By contrast, Catlin et al. reported a median T/E ratio of 1.1 in a sample of 3710 football players (Catlin et al., 1997). This is in nearly perfect agreement with our data and demonstrates the importance of using representative populations as a starting point for developing an adaptive model as well as for establishing population based reference limits.

Table 2 – Summary statistics for individual samples

Parameter	N	Mean	Std Dev	Min	Median	99th %	99.9th %	Max
Corrected T/E	17030	1.30	1.01	0.00	1.06	5.02	7.31	11.69
Testosterone	13038	36	20.50	6.00	31.0	106	159	256
Epitestosterone	16391	38	23.8	1.00	33.00	116	169	307
Androsterone	16714	2782	1383	59	2540	7245	10885	17600
Etiocholanolone	16709	1980	1034	58	1764	5533	8503	12952
DHEA	15485	37	22.4	5	32	119	189	278
Andro/Etio	16671	1.56	0.72	0.03	1.43	3.81	5.92	9.69
Andro/Test	12964	100.8	64.7	3.38	85.4	350	517.3	655.9

Figs. 1-6 show individual parameter distributions of T/E ratio, A/E ratio, Test, EpiT, Andro and Etio. Note the bimodal distribution of T/E ratio which is indicative of a minority population of UGT 2B17 double deletion polymorphism. (Jakobsson et al., 2006) This bimodal distribution and its relative proportions of "low" and "normal" basal T/E ratio has been well documented by other groups (Ayotte et al., 1996; Catlin et al., 1997; Sottas et al., 2007; Van Renterghem et al., 2010). This bimodal distribution is not seen in the graph of T concentrations because the limit of quantitation (LOQ) of the assay used for these analyses was 10 ng/ml, which resulted in the exclusion of values below that threshold. By contrast, WADAs protocol for reporting T/E ratios is based upon chromatographic peak area only and is not subject to LOQs. The other graphs show fairly typical distributions with slight skewing toward elevated values.

Figure 1

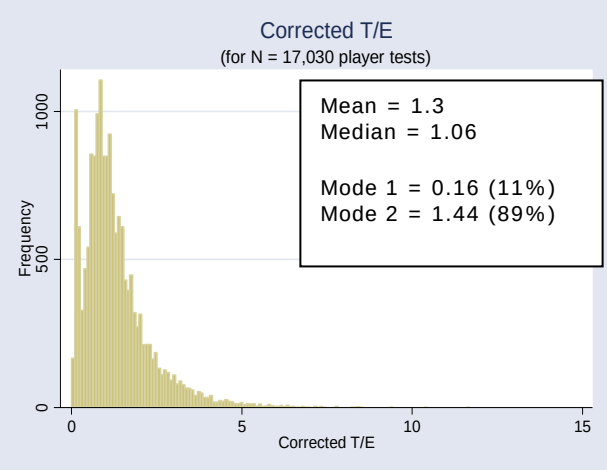


Figure 2

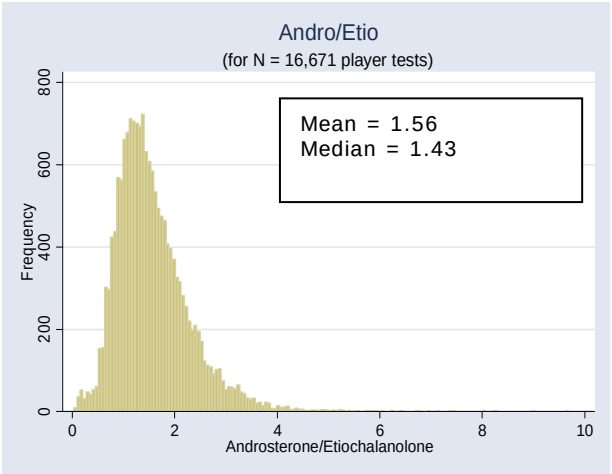


Figure 3

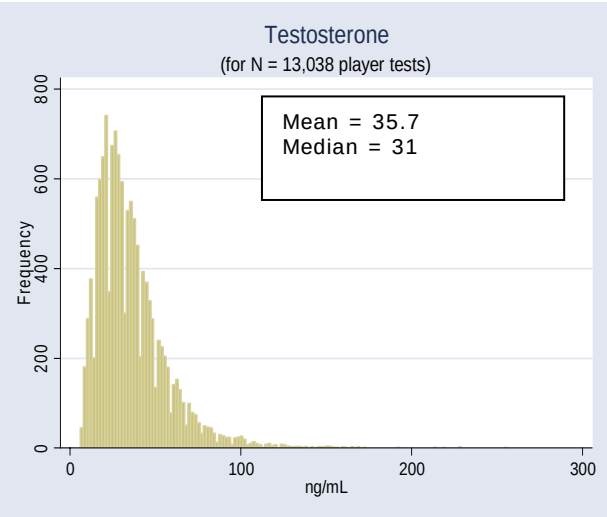


Figure 4

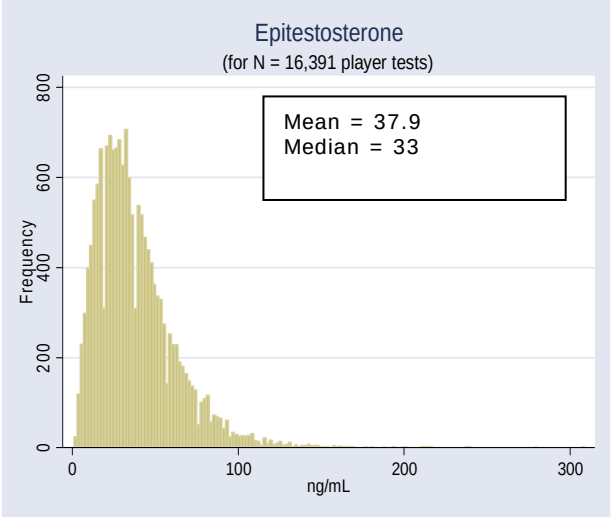


Figure 5

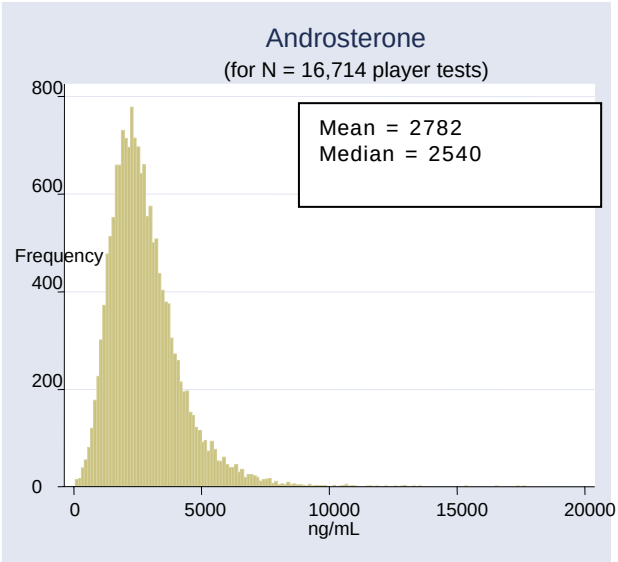
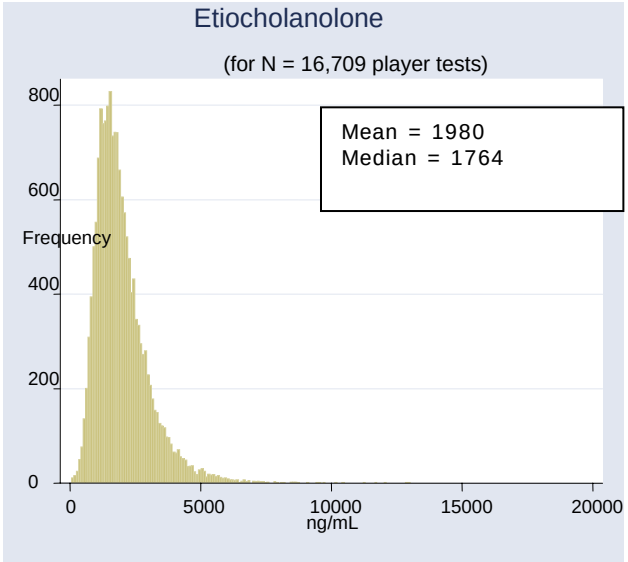


Figure 6



While individual tests are appropriate for determining population reference limits, we do not have the same number of tests for each individual. In order to avoid disproportionately biasing our data, we also calculated summary statistics by player, in which each player's mean was used as an individual data point. This data is shown in Table 3. Direct comparison demonstrates that while the minimum and maximum values, as well as some standard deviations are obviously attenuated, there is no appreciable difference between the means or medians of the two data sets, indicating that there does not seem to be any significant bias associated with using individual samples for our database.

Table 3 – Summary statistics for player means

Variable	N	Mean	Std Dev	Min	Median	99th Percentile	Max
Corrected T/E	3073	1.30	0.96	0.05	1.08	4.96	8.09
Testosterone	2762	33.7	17.05	6.00	30.5	90.00	156
Epitestosterone	3054	36.9	20.32	2.00	34.0	99.50	168
Androsterone	3075	2832	1124	227	2658	6609	11511
Etiocholanolone	3074	2005	882	346	1832	4913.4	9572
DHEA	3038	37.5	17.7	5.00	33.7	95.50	192
Andro/Etio	3074	1.57	0.63	0.13	1.47	3.43	7.12
Andro/Test	2759	110.7	66.1	3.47	95.45	367.2	614.5

One issue that is of fundamental importance in establishing individual reference limits is an assumption that individuals have smaller variances than populations. Because the variables in the steroid profile have a wide range of absolute values, it is common to use coefficients of variation (CVs), or the value of the mean over standard deviation (μ/σ) to measure the relative variance of individuals. Previous studies show that individual CVs are fairly narrow compared with population variances (Donike et al., 1993; Mareck-Engelke et al., 1994) and WADA guidelines suggest that a CV of greater than 30% for T/E is indicative of exogenous steroid use. Table 5 shows the mean CVs of each parameter in our database. Regression analysis reveals that the mean and CV are independent for these variables. Fig. 7 shows the plot of mean T/E ratio vs. CV for 3073 athletes. The fitted linear regression has an R^2 value of 0.0066 indicating the independence of the relationship. Fig. 8 demonstrates that the CVs for T/E are fairly well distributed around the mean of 24%.

Table 5 – Mean CV and R^2 for mean vs. CV

	T/E	A/E	A/T	T	E	Andro	Etio	DHEA
Mean CV	23.76%	25.00%	38.18%	35.96%	37.42%	35.59%	33.70%	42.73%
R^2 - mean vs. CV	0.0066	0.0012	0.0061	0.0375	0.0764	0	0.0218	0.083

Figure 7

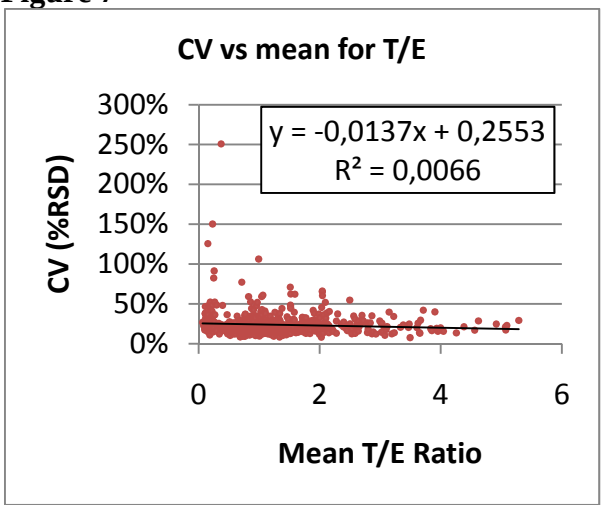
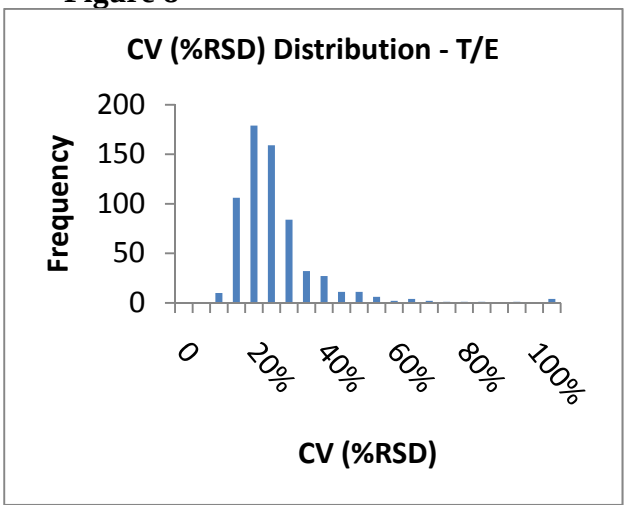


Figure 8



Conclusions

We have used a database of nearly 17000 tests from 3100 athletes to determine population statistics for the endogenous steroids and ratios in NFL players analyzed over a two year span in our laboratory. This is the first time that this type of analysis has been performed on steroid parameters other that T/E in this population. The data are in consistent with previous studies in NFL players. Comparison with other populations reveals many similarities, such as the bimodal T/E distributions, as well as some differences that may be attributable to either ethnicity, athlete body type, or other factors. We have also shown that these steroid parameters have relatively small coefficients of variation that is not related to the magnitude of the measurement, an important requirement for the adaptive models we are evaluating. These data represent a valuable asset that we can build upon and use for further steroid profiling research.

Acknowledgements

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