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Endogenous Nandrolone Metabolites in Human Urine. Two-Year Monitoring of Male Professional Soccer Players

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Abstract

19-Norandrosterone (19-NA) and 19-noretiocholanolone (19-NE) are the two main indicators used to prove the illegal use of nandrolone by humans. Recent studies showed that 19-NA and 19-NE can be endogenously produced in some individuals. The mediated cases observed over the last three years generated some questions about the appropriateness of the official International Olympic Committee cutoff level, which is 2 ng/mL of 19-NA in male urine samples. In the present study, professional soccer players belonging to the French First League were studied over a period of 19 months. In total, 385 urine samples were taken immediately before and after soccer competitions and were coupled with 200 blood samples for testosterone and LH determination. Results of the study showed that the mean values for 19-NA and 19-NE were 0.097 ng/mL and 0.033 ng/mL, respectively. For 19-NA, 70% of the samples proved to be below 0.1 ng/mL, whereas less than 20% were found to be between 0.1 and 0.2 ng/mL, and 7% were between 0.2 and 0.3 ng/mL. Only four urine samples were above 1.0 ng/mL; the maximal value was 1.79 ng/mL. For 19-NE, only one sample was above 1.0 ng/mL; the value was 1.42 ng/mL. Concentrations of these compounds after games were generally significantly higher than those before games.

Introduction

Nandrolone (19-nortestosterone, 19-NT), when administered to humans, is known to be converted into two main metabolites, 19-norandrosterone (19-NA) and 19-noretiocholanolone (19-NE). These two degradation products are also used to prove the illegal use of 19-NT or other precursors such as 19-norandrostenediol or 19-norandrostenedione; the International Olympic Committee (IOC) cutoff levels in urine for these metabolites are fixed at 2 ng/mL for men and 5 ng/mL for women. In 1997, a debate about the capability of the human body to endogenously produce traces of 19-NA and 19-NE was initiated after the systematic discovery of low concentrations of these metabolites in urine. These findings were essentially due to the introduction in control laboratories of hyphenated analytical techniques such as gas chromatography coupled with high-resolution mass spectrometry (GC–HRMS) in the selected ion monitoring (SIM) mode or tandem mass spectrometric (MS–MS) techniques in the selected reaction monitoring (SRM) mode, techniques which are particularly sensitive and specific. The endogenous production of 19-NA and 19-NE has been reported by the scientific community since 1998 (1–7). The endogenous concentrations obtained from a population of non-athlete individuals showed values contained in-between the method detection limit (often below 0.1 ng/mL) and 0.60 ng/mL (2–4,7). An athletic effort was suggested as a potential etiology for increased nandrolone metabolite concentration in urine, but this was not confirmed on a large scale. A recent statistical study (8) was conducted with players from the first and second divisions of the Swiss soccer national league. Results showed that without physical activity, no metabolites of nandrolone were detectable (amateur and students), and that after soccer games, metabolites appeared in 6% of the population tested at a concentration between 0.2 and 2.0 ng/mL. Less than 1% of the study population excreted concentrations above 2 ng/mL. No complementary tests (LH and testosterone in blood) were done to exclude potential nandrolone administration, and for practical reasons, no samples were taken before athletic activity. Moreover, food intake by this population at least 24 h before competition was not controlled, and no correlation was performed between individual general data (such as age or weight) and excretion level. At last, the observation of the athletes was not spread over a large period of time. The objective of the present paper was to add complementary data to the study previously described (8) on the basis of the study of 40 professional football players for a period of one and one-half years.

Experimental

All reagents and solvents were of analytical-grade quality and provided by Solvants Documentation Synthesis (SDS, Peypin,

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France). The derivatization reagents *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) and trimethyliodosilane (TMIS) were purchased from Fluka (Buchs, Switzerland). Dithiothreitol (DTT) was from Aldrich (Milwaukee, WI). β-Glucuronidase (*E. coli*) was provided by Boehringer (St. Quentin Fallavier, France). Thirty-six soccer players and four trainers belonging to the Football Club de Nantes Atlantique (FCNA, French Soccer First League) participated to the study for one year and a half (26 October 1998 to 23 May 2000). Thirty-six healthy male volunteers (18–32 years old) and four trainers (36–52 years old) agreed to submit urine samples. All human subjects signed written, informed consent. After collection, urine samples were stored below –18°C. The urine samples (385) were taken immediately before and after soccer games, and they were coupled with 200 blood samples for testosterone and LH determination. Food intake was controlled by the team physician at least 24 h before competition. Nevertheless, food was never controlled to check for contamination by steroid compounds. Rough data concerning the participants age, weight, size, race, behavior, and fitness were recorded, and sampling was spread over the whole study period. After fortifying with 3 ng 19-NE-d_3 (Radiant-Promochem, Molsheim, France), 10 mL urine were hydrolyzed (1 h, 52°C, pH 6.5) in the presence of 2 mL phosphate buffer (0.1M, pH 6.5) and 80 mL β-glucuronidase from *E. coli*. Each sample was then applied onto a C18 column, previously conditioned with 10 mL methanol and 10 µL water. Steroid analytes were eluted with 5 mL of methanol/ethyl acetate (30:70, v/v). The eluting fraction was washed twice with 2 mL of 1M sodium hydroxide. After evaporation, the dry residue was resuspended in hexane/dichloromethane (60:40, v/v) and applied to a silica SPE column. After washing the stationary phase with 8 mL hexane/ethylacetate (85:15, v/v), analytes were eluted with 13 mL hexane/ethylacetate (60:40, v/v). After evaporation, the dry residue was reconstituted in 30 mL methanol/water (40:60, v/v) before injection onto a C18 HPLC column (15 × 0.2 cm, 5-µm particle size, Zorbax, Interchim, France). The mobile phase was methanol/water (40:60, v/v at t = 0 min, 90:10, v/v at t = 15 min for 5 min, 100:0, v/v at t = 25 min). The collected fraction was dried and derivatized for 40 min at 60°C with MSTFA/TMIS/DTT (1000:5:5, v/v/w). The quadrupole MSs used were 5970 and 5973 coupled to 5890 and 6890 GCs, respectively; both were acquired from Hewlett-Packard (Palo Alto, CA). Ionization was performed under electronic impact (EI) conditions, and data acquisition was done in the SIM mode (*m/z* 423 and 408 for 19-NE-d_3 and *m/z* 420, 405, 315, and 225 for 19-NA and 19-NE).

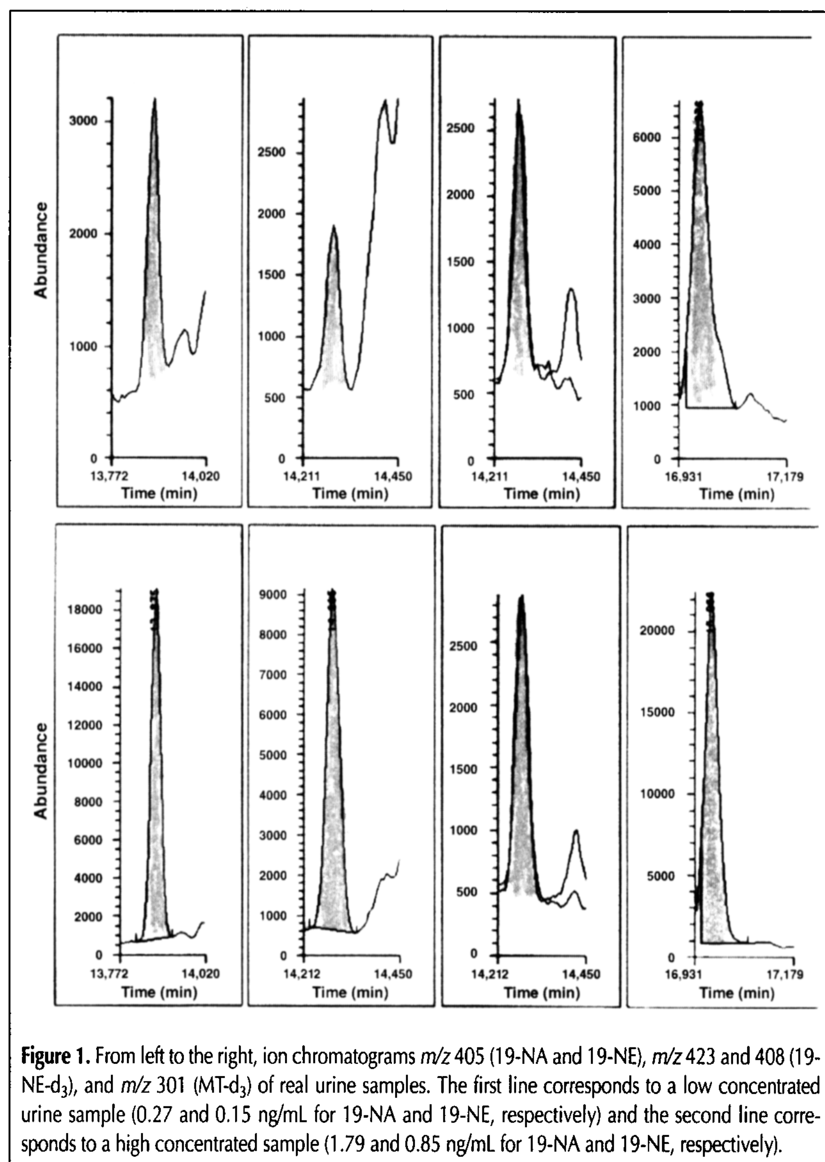

Figure 1. From left to the right, ion chromatograms *m/z* 405 (19-NA and 19-NE), *m/z* 423 and 408 (19-NE-d_3), and *m/z* 301 (MT-d_3) of real urine samples. The first line corresponds to a low concentrated urine sample (0.27 and 0.15 ng/mL for 19-NA and 19-NE, respectively) and the second line corresponds to a high concentrated sample (1.79 and 0.85 ng/mL for 19-NA and 19-NE, respectively).

Results and Discussion

It is obvious that the approach consisting of a rapid and non-specific purification becomes rapidly not acceptable, especially when the concentration of the test compound is low. Even when hyphenated techniques are used (MS–MS or HRMS), an insufficient preparation can be a handicap when compound levels are down to 0.5 ng/mL. More sophisticated purification techniques, such as complementary solid-phase extraction purifications coupled to preparative HPLC, appear to be a necessary solution to reach the 10-pg/mL detection level. Moreover, it is important to emphasize that quantitative data given hereinafter correspond to the sum of free and glucuronidated forms of nandrolone metabolites. Finally, no concentration correction was performed on the basis of the specific gravity or creatinine content of the urine, in spite of the current procedure performed in IOC laboratories when the specific gravity exceeds 1.020 (application of the IOC Medical Commission Document, August 7th 1998).

Validation

The limit of detection was evaluated on the measurement of 25 buffer samples fortified with 0.2 ng/mL 19-NA and 19-NE. At a signal-to-noise ratio equal to 3, the detection limit on *m/z* 405

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signal was 0.02 and 0.03 ng/mL for 19-NA and 19-NE, respectively. Extraction repeatability was evaluated on the basis of ten samples fortified at 0.2 ng/mL and analyzed the same day by the same operator. Repeatability was 16.2% and 8.0% for 19-NA and 19-NE, respectively. Extraction reproducibility was assessed on 25 samples fortified at 0.2 ng/mL and analyzed over 4 days by the same operator. Reproducibility was 18.9% and 15.4% for 19-NA and 19-NE, respectively. Better 19-NE results were obtained for both repeatability and reproducibility from 25 samples fortified at 0.2 ng/mL; 11.6% and 10.9% were calculated for 19-NA and 19-NE, respectively. These low absolute values were the consequence of the four purification steps, which were absolutely necessary to avoid false-positive results (2,7). Calibration curves were performed daily over the 4 days using a blank and 0.15, 0.30, and 0.45 ng/mL fortified samples. For 19-NA, the determination coefficient (R^2) varied within the 0.959 and 0.998 range, whereas values for 19-NE were between 0.966 and 1.000. Slopes were within 9.067–11.778 and 6.972–8.802 ranges for 19-NA and 19-NE, respectively. Precision (RSD) was 11.5% and 10.7% for 19-NA and 19-NE, respectively (25 fortified samples at 0.2 ng/mL).

Analytical strategy and performance

Each sample batch contained three fortified samples dedicated to the calibration curve determination (0.15, 0.30, and 0.45 ng/mL), a blank sample, one standard, and no more than 20 test samples. 19-Noretiocholanolone-d_3 was used as the internal standard. SIM acquisition was performed on *m/z* 420, 405, 315, and 225 for nandrolone metabolites, and on *m/z* 423 and 408 for the internal standard; *m/z* 405 was used to quantitate 19-NA and 19-NE. Certain analytical criteria were checked before taking the batch into consideration (internal standard S/N, recovery yield, and calibration curve linearity). All samples exceeding 0.3 ng/mL in 19-NT metabolites were extracted again for confirmation of the concentration. For this purpose, the composition of the batch consisted of four fortified samples dedicated to the calibration curve determination (0.20, 0.80, 1.40, and 2.00 ng/mL for 19-NA and 0.15, 0.30, 0.45, and 0.60 ng/mL for 19-NE), a blank sample, one standard, and no more than 20 test samples. Examples of real urine samples are given in Figure 1. Almost no interference occurred in the neighboring region of the expected retention time of the *m/z* 405 and 408 ion chromatograms. The risk of false-positive results (i.e., to declare one analyte present when it is not) was low.

Quantitative results

Concentration mean values (i.e., whatever the sampling moment, before or after) were 0.097 ng/mL and 0.033 ng/mL for 19-NA and 19-NE, respectively. Concentrations obtained for 19-NA were 0.068 and 0.136 ng/mL before and after games, respectively. For 19-NE, the respective mean concentrations were 0.015 and 0.057 ng/mL before and after physical effort.

Cases of non-detection. The non-detection of 19-NA compared with 19-NE was less frequent. Indeed, in 13.5% of the samples, 19-NA was below the detection limit (0.02 ng/mL), whereas

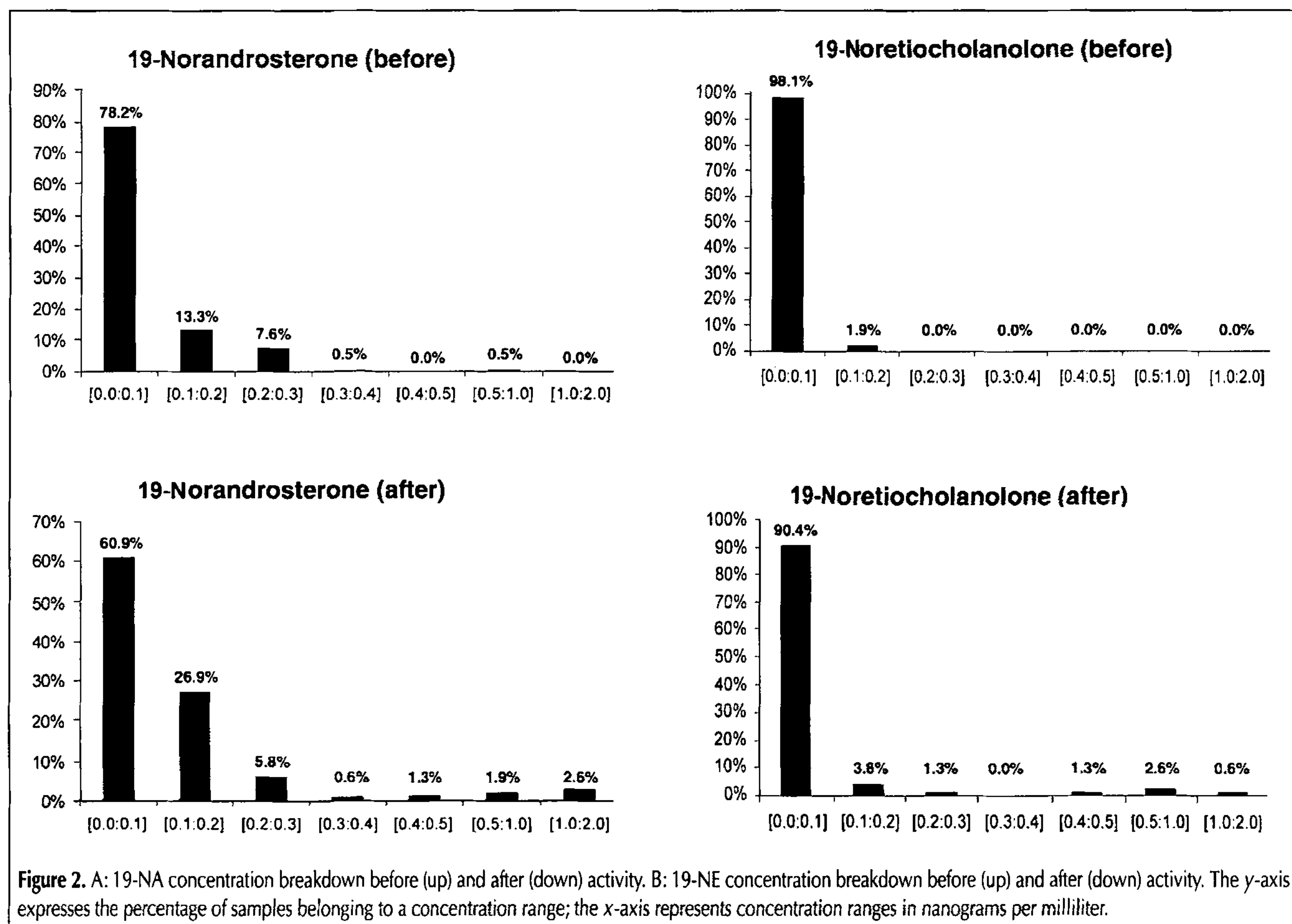

Figure 2. A: 19-NA concentration breakdown before (up) and after (down) activity. B: 19-NE concentration breakdown before (up) and after (down) activity. The *y*-axis expresses the percentage of samples belonging to a concentration range; the *x*-axis represents concentration ranges in nanograms per milliliter.

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38.2% of urine samples could not be shown to contain any residue of 19-NE (LOD 0.03 ng/mL). Non-detection was more frequent before games than after, regardless of the metabolite considered: 19-NA (16% before, 10% after) and 19-NE (43% before and 31% after).

Breakdown of the population by concentration group. A summary of the results for 19-NA and 19-NE concentrations is given in Figure 2. For 19-norandrosterone, 70.8% of urine samples were found to be below 0.1 ng/mL, 19.8% were between 0.1 and 0.2 ng/mL, 6.8% were in the range between 0.2 and 0.3 ng/mL, and 3.2% were above 0.3 ng/mL. Among the latter, only 1% (4 samples) was higher than 1.0 ng/mL. No urine samples exceeded the IOC cutoff level, which is 2.0 ng/mL. The highest value detected was 1.79 ng/mL.

Consequences of physical activity. Of the samples collected before physical activity, 99% were found to be below 0.3 ng/mL; only two samples had higher concentrations (0.30 and 0.80 ng/mL). After the games, the breakdown of the population by concentration group was clearly changed: 6.4% were above 0.3 ng/mL. It is obvious that concentrations of nandrolone metabolites are more important after competition than before. With respect to 19-noretiocholanolone, 94.8% of urine samples were found below 0.1 ng/mL, 4.8% were between 0.1 and 1.0 ng/mL, and 0.3% (one urine sample) was above 1.0 ng/mL (1.42 ng/mL); none exceeded the IOC decision criteria. Once again, all of the samples with the highest concentrations were collected after the soccer competition. Differences in concentration before and after games observed on the five urine samples containing the highest nandrolone metabolite contents are shown in Figure 3. The metabolite concentration before games was close to the limit of detection (0.02 ng/mL), and immediately after games, it was above 1 ng/mL in some cases.

19-NA-to-19-NE ratio. The calculation of the ratio between 19-NA and 19-NE was difficult to evaluate especially for the lowest concentrations. Among the most concentrated samples (Table I), the ratio between 19-NA and 19-NE showed that in almost all the cases, 19-NA was at least as abundant as 19-NE. The 19-NA to 19-NE ratio varied in general between 1.5 and 3 with a mean value close to 2. This ratio was not significantly different from the one observed when nandrolone or one of its precursors was used (9,10). No conclusion can be drawn on this ratio to discriminate between endogenous production and exogenous administration.

Correlation between concentrations and sample characteristics. Most of the individuals tested did not produce 19-NA or 19-NE above 0.3 ng/mL. Seven out of the 36 players appeared to be more predisposed to excrete high concentrations of nandrolone metabolites than the others (Table I). It was the case of player "8" (171 cm, 73 kg, Caucasian type, forward) and player "6" (171 cm, 67 kg, African type, midfield). We wondered whether player position on the field influenced nandrolone production; defender, midfield, and forward were considered, but goalkeeper was not.

No evident differences were found between seasons, with one exception. Indeed, among the 13 highest concentrated urine samples (Table I), 7 were observed during the "F" sampling period corresponding to the end of April 1999. This soccer match involved a French first league championship game played at home in front of 26,000 spectators with fine weather. The final result was 0–0, and only one player received a yellow card.

Some complementary investigations were performed to check the incidence of stress on the quantitative production of nandrolone metabolites. The testing was done on trainers during a soccer match of the French Cup characterized by an extra time period and penalties. No significant increase was observed among the individuals tested, and concentrations remained in between the limit of detection and 0.05 ng/mL.

Moreover, we wondered whether the level of game influenced the production of 19-NA and 19-NE. Of the most concentrated urine samples (Table I), nine came from individuals playing in the

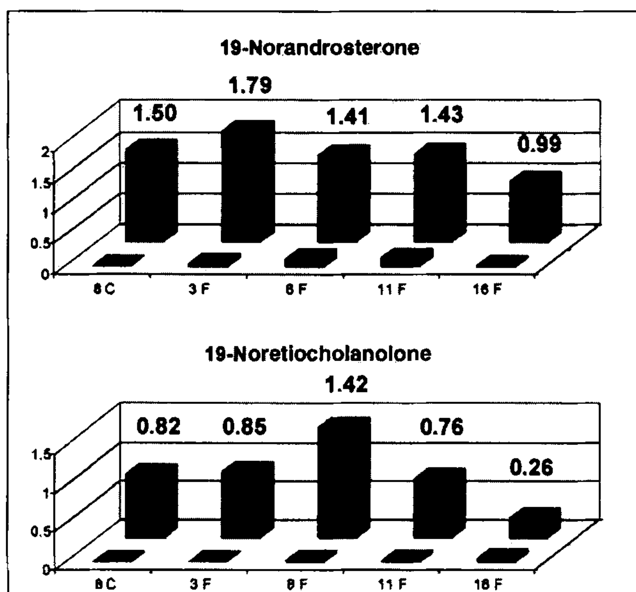

Figure 3. 19-NA and 19-NE urinary concentrations before and after exercise. The *y*-axis expresses the concentration of each nandrolone metabolite in nanograms per milliliter; individual urine sample identification is shown on the *x*-axis.

Table I. Concentrations of the Thirteen Urine Samples Containing the Highest Content of Nandrolone Metabolites*

Sample	19-NA	19-NE	NA/NE
3FII	1.79	0.85	2.1
8CII	1.50	0.82	1.8
12FII	1.43	0.76	1.9
8FII	1.41	1.42	1.0
16FII	0.99	0.26	3.8
9FII	0.86	0.40	2.2
13BII	0.83	0.43	1.9
24FII	0.43	0.73	0.6
8EII	0.42	0.18	2.3
8ZII	0.37	0.12	3.1
6DI	0.27	0.15	1.8
37GI	0.24	0.16	1.5
6FII	0.23	0.08	2.9

* The first column indicates sample identification (first number is player identity, second letter is the collecting period, last letters indicates before (I) or after (II) game), the second and third columns report concentrations in ng/mL of 19-NA and 19-NE, respectively, and the last column corresponds to the ratio.

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first league, and two were from individuals playing in CFA2 (first level of national amateur championship). This evidence did not suggest any effect of level of play on 19-NT metabolite production.

Finally, we tried to correlate increased production of nandrolone with the behavior of the individual on the field in terms of sending off, warnings, and physical or verbal aggressiveness. No significant relationship between these parameters was observed.

Conclusions

It is still difficult after this study to be more precise about the production origin and pathway of 19-NA and 19-NE. Nevertheless, it can be asserted that the presence of these metabolites was not due to any diet contamination (e.g., pork (11), horse, and energetic tablets...) or steroid misuse. The dietary factor was dismissed because the professional soccer group was isolated for at least 24 h before the sporting event, and all of the menus were planned and under the control of the team practitioner. On the other hand, all unilateral potential doping was controlled through LH and testosterone measurements in blood. The 40 participants were checked 3 times, and no perturbation was observed during the study period. In summary, it was observed that 100% of the 385 urine samples tested were below the IOC cutoff level, and the highest value was 1.79 ng/mL. No concentration correction was applied (neither specific gravity nor creatinine concentration) in spite of the IOC Medical Commission guidelines usually used by official control laboratories. The highest concentrations of metabolites were found almost exclusively in urine samples collected after sporting events. Moreover, the ratio between 19-NA and 19-NE did not permit for the unambiguous differentiation between an endogenous and exogenous presence. With respect to the parameters in which high concentrations were observed, it appeared that some individuals were more affected. For example, it was remarked that during one sampling period (end of April 1999), concentrations were significantly higher compared with those observed during all of the other sample periods. On the other hand, it was not possible to correlate stress, level of game (First League, Amateur league), or aggressive behavior of the individual during the sporting event with increased excretion of 19-nortestosterone metabolite in urine. A study of the nandrolone phase II metabolite composition among the highest concentrated urine samples was conducted. The preliminary results, which constitute a new approach for the discrimination between normal and illegal presence, has been published elsewhere (12).

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References

1. B. Le Bizec and F. André. New approach for antidoping laboratories: evidence for 19-norandrosterone and 19-noretiocholanolone endogenous production in man. Presented at the 15th French Mass Spectrometry Symposium, Lyon, France, September 8–10, 1998.
2. B. Le Bizec, F. Monteau, I. Gaudin, and F. André. Evidence for the presence of endogenous 19-norandrosterone in human urine. *J. Chromatogr. B* **723:** 157–172 (1999)
3. L. Dehennin, Y. Bonnaire, and P. Plou. Urinary excretion of 19-norandrosterone of endogenous origin in man: quantitative analysis by gas chromatography–mass spectrometry. *J. Chromatogr. B* **721:** 301–307 (1999).
4. U. Mareck-Engelke, H. Geyer, and W. Schänzer. 19-Norandrosterone—criteria for the decision making process. Recent advances in doping analysis (6). Proceedings of the Manfred Donike Workshop 16th Cologne Workshop on dope analysis 15th to 20th March 1998, W. Schänzer, H. Geyer, A. Gotzmann, and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, Germany, 1999, pp 119–129.
5. M. Ciardi, R. Ciccoli, M.V. Barbarulo, and R. Nicoletti. Presence of norandrosterone in "normal" urine samples. Recent advances in doping analysis (6) Proceedings of the Manfred Donike Workshop 16th Cologne Workshop on dope analysis 15th to 20th March 1998, W. Schänzer, H. Geyer, A. Gotzmann, and U. Mareck-Engelke, Eds. Sport und Buch Strauss, Köln, Germany, 1999, pp 97–104.
6. P. Kintz, V. Cirimele, and B. Ludes. Norandrosterone and noretiocholanolone: metabolite markers. *Acta Clin. Belg.* Suppl **1:** 68–73 (1999).
7. B. Le Bizec, I. Gaudin, A. Pohu, F. Monteau, and F. André. Identification of endogenous 19-norandrosterone in human urine. Recent advances in doping analysis (7). Proceedings of the Manfred Donike Workshop 17th Cologne Workshop on dope analysis 14th to 19th March 1999, W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke Eds. Sport und Buch Strauss, Köln, Germany, 2000, pp 109–119.
8. M. Saugy, N. Robinson, C. Cardis, C. Schweizer, L. Rivier, P. Mangin, C. Ayotte, and J. Dvorak. Nandrolone metabolites in football players: utility for in and out of competition tests. Recent advances in doping analysis (7). Proceedings of the Manfred Donike Workshop 17th Cologne Workshop on dope analysis 14th to 19th March 1999, W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck-Engelke Eds. Sport und Buch Strauss, Köln, Germany, 2000, pp 95–107.
9. W. Schänzer. Metabolism of anabolic androgenic steroids. *Clin. Chem.* **42(7):** 1001–1020 (1996).
10. W. Schänzer. Metabolism of anabolic androgenic steroids: 5α- and 5β-reduction of 3-keto-4-ene steroids. Recent advances in doping analysis (5). Proceedings of the Manfred Donike Workshop 14th Cologne Workshop on dope analysis 17th to 22nd March 1996, W Schänzer, H Geyer, A Gotzmann and and U Mareck-Engelke Eds. Sport und Buch Strauss, Köln, Germany, 1997, pp 185–201.
11. B. Le Bizec, I. Gaudin, F. Monteau, F. André, S. Impens, K. de Wasch, and H. De Brabander. Consequences of boar edible tissue consumption on urinary profiles of nandrolone metabolites. I. Mass spectrometric detection and quantification of 19-norandrosterone and 19-noretiocholanolone in human urine. *Rapid. Commun. Mass Spectrom.* **14(12):** 1058–1065 (2000).
12. B. Le Bizec, F. Bryand, I. Gaudin, F. Monteau, F. Poulain, and F. André. Endogenous nandrolone metabolites in human urine. Preliminary results to discriminate between endogenous and exogenous presence. *Steroids* **67(2):** 105–110 (2002).

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