



# **THE EFFECT OF MUSCULOSKELETAL INJURY ON ENDOGENOUS NANDROLONE METABOLISM**

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## List of abbreviations

▪ Adrenocorticotrophic hormone	ACTH
▪ Anabolic androgenic steroids	AAS
▪ Body Mass Index	BMI
▪ Dehydroepiandrosterone	DHEA
▪ Gas chromatography isotope ratio mass spectrometry	GC- C-IRMS
▪ Gas chromatography mass spectrometry	GC-MS
▪ Hypothalamic pituitary axis	HPA
▪ Injury severity score	ISS
▪ International Olympic Committee	IOC
▪ Level Of Detection	LOD
▪ Luteinizing hormone	LH
▪ mass and charge	m/z
▪ nanogram per milliliter	ng.ml <sup>-1</sup>
▪ 19-norandrosterone	19-NA
▪ 19-noretiocholanolone	19-NE
▪ picogram per milliliter	pg.ml <sup>-1</sup>

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## Abstract

Two elite international male athletes tested positive for nandrolone after both sustaining a severe musculoskeletal injury. Both athletes denied the intentional use of nandrolone. No previous study has investigated the effect that a severe physiological stress in the form of musculoskeletal injury can have on the excretion of nandrolone metabolites (19-NA and 19-NE) in the urine of athletes. Accordingly, the first phase of this thesis involved a review of the literature. This review showed that there is a theoretical basis for the excretion of nandrolone metabolites in the urine to be altered by various physiological stimuli. Therefore the main aim of the thesis was to validate the International Olympic Committee's cut-off concentration for nandrolone metabolites (19-NA and 19-NE) in the urine of male athletes ( $2000 \text{ pg}\cdot\text{ml}^{-1}$ ). Other aims of the thesis were to determine if musculoskeletal injury can increase the concentration of nandrolone metabolites in the urine of normal subjects and to confirm the endogenous production of 19-NA and 19-NE in the urine of athletes free of exogenous nandrolone. The results were that one of the injured subjects, free of synthetic nandrolone, produced a 19-NA concentration of  $2800 \text{ pg}\cdot\text{ml}^{-1}$  (IOC cut-off =  $2000 \text{ pg}\cdot\text{ml}^{-1}$ ). This finding represents a false positive doping test for nandrolone and will require further similar studies to validate this finding. Also, musculoskeletal injury did not result in a significant increase in 19-NA or 19-NE concentrations in the urine when compared to baseline. This study confirms the endogenous production of small amounts of 19-NA and 19-NE in subjects not administering synthetic nandrolone. In accordance with this, multi-centre studies need to further answer specific questions regarding the current urine threshold concentrations for nandrolone metabolites and whether physiological stressors like musculoskeletal injury can affect nandrolone metabolite excretion.

**Key words:** nandrolone, aromatisation, musculoskeletal injury, injury severity score, 19-norandrosterone (19-NA), 19-noretiocholanolone (19-NE)

## Chapter 1

### Motivation and aims of the thesis

#### 1.1 Introduction

Nandrolone (19-nortestosterone) is a derivative of the naturally occurring anabolic steroid hormone testosterone found in humans. Testosterone is important in the development of secondary sexual characteristics in males.<sup>29</sup> Nandrolone was first synthesized in 1950<sup>51</sup> and has become a popular anabolic androgenic steroid (AAS) amongst sportspersons.<sup>32;89</sup> Nandrolone is reportedly able to increase lean muscle mass and improve strength gains, thereby enhancing athletic performance.<sup>67</sup> At the same time, very few significant side effects have been reported, thus contributing to its popularity. The International Olympic Committee (IOC) banned the use of nandrolone in sporting competition in 1976 and in 1985 introduced gas chromatography mass spectrometry (GC-MS) with a level of detection of  $2 \text{ ng.ml}^{-1}$  to test for the metabolites (breakdown products) of nandrolone in the urine of athletes. The three metabolites of nandrolone are 19-norandrosterone (3 alpha-hydroxy-5 alpha-estran-17-one), 19-noretiocholanolone (3 alpha-hydroxy-5 beta-estran-17-one) and 19-norepiandrosterone (3 beta-hydroxy-5 alpha-estran-17-one).<sup>5;7;17;59;67;78</sup> At that time the detection of any nandrolone metabolite in the urine of an athlete was considered to constitute a doping offence. In 1996, more sensitive testing was introduced at the Winter Olympic Games in Nagano, Japan, using a device capable of measuring 19-norandrosterone (19-NA), the most abundant metabolite of nandrolone<sup>5;17;59;67;78</sup>, to a level of detection  $0.02 \text{ ng.ml}^{-1}$ .<sup>36</sup> At this time, the IOC issued a statement announcing that 19-NA concentrations in the urine of male subjects below  $2 \text{ ng.ml}^{-1}$  would not constitute a doping offence.<sup>82</sup> This decision may have been based on the finding of a

small amount of 19-NA in the urine of many athletes at the Nagano Winter Olympics who had not used nandrolone. Therefore, a cut-off concentration in the urine for 19-NA of  $2 \text{ ng.ml}^{-1}$  in male athletes and  $5 \text{ ng.ml}^{-1}$  in female athletes was established.<sup>82</sup> It should be noted that this decision was taken by the IOC prior to any controlled scientific studies being performed to validate the cut-off concentration of 19-NA in the urine. This became important when subsequent independent scientific studies confirmed the endogenous production of small amounts of 19-NA in the urine of resting and athletically active populations.<sup>10;20;28;28;43;48;51;72;75;79;93</sup> The origin of the endogenous 19-NA may be the aromatisation reaction of testosterone to estrogen.<sup>7;10;20;21;40;62;72;73;86;90;93</sup> Further, Reznik et al (2001) has shown that this aromatisation reaction can be upregulated by administering human chorionic gonadotropin which increased the concentration of 19-NA in the urine of subjects.<sup>72</sup> Therefore, it is relevant to establish whether any physiological processes in the human body, which can potentially affect testosterone metabolism and aromatisation, may affect the production and excretion of endogenous 19-NA. These possibilities were not considered by the IOC when deciding on the cut-off concentration of  $2 \text{ ng.ml}^{-1}$  in male subjects. Various physiological processes have been proposed as having an effect on the excretion of nandrolone metabolites in the urine. Certain forms of exercise have the potential to affect testosterone metabolism.<sup>45;46;55;80;87;94;95</sup> The results of studies investigating the effect of exercise on the excretion of 19-NA in the urine have showed mixed results. It seems that the intensity of the exercise may be an important factor in predicting whether exercise will increase the excretion of 19-NA in the urine.

28;48;51;75;79

Recently, two high-profile nandrolone doping cases involved athletes who had sustained severe musculoskeletal injury before passing a urine sample. This has raised the possibility that severe physiological stress in the form of musculoskeletal injury may affect the excretion of 19-NA in the urine of athletic individuals free of synthetic nandrolone. <sup>64</sup>(Personal communication) No previous study has investigated whether a severe musculoskeletal injury stress can increase the excretion of nandrolone metabolites in the urine of male subjects.

## 1.2 Aims of the thesis

- To validate the International Olympic Committee's cut-off concentration for urine 19-NA of  $2 \text{ ng.ml}^{-1}$  in male subjects immediately after sustaining musculoskeletal injury.
- To determine whether moderately active male subjects who sustain severe physiological stress (musculoskeletal injury) can excrete nandrolone metabolites (19-NA and 19-NE) in the urine in higher concentrations compared to baseline.
- To confirm the endogenous production of 19-NA and 19-NE in the urine of moderately active male subjects.

## 1.3 Research question

*"Can severe musculoskeletal injury in healthy moderately active male subjects increase the excretion of endogenous nandrolone metabolites in the urine above  $2 \text{ ng.ml}^{-1}$ ?"*

## Review of endogenous nandrolone metabolism

(Br J Sports Med 2002; 36:325-329)

### 2.1 Introduction

The anabolic androgenic steroid 19-nortestosterone, also called nandrolone, was first synthesized by Birch in 1950.<sup>51</sup> Nandrolone has an anabolic effect, and is used in the treatment of certain chronic diseases.<sup>22;51;76</sup> The use of nandrolone by athletes became popular in the late 1950's.<sup>32</sup> Athletes use nandrolone in an oral or injectable form to increase muscle strength and improve performance.<sup>47</sup> As a result of the potential performance enhancing benefits<sup>89</sup> and potential health risks associated with anabolic steroid use,<sup>34</sup> the International Olympic Committee (IOC) prohibited the use of nandrolone in sport in 1976.

When nandrolone is ingested or injected by human subjects, three metabolites of nandrolone are isolated and measured in the urine using gas chromatography-mass spectrometry (GC-MS). These metabolites have been identified as 19-norandrosterone (3 alpha-hydroxy-5 alpha-estran-17-one), 19-noretiocholanolone (3 alpha-hydroxy-5 beta-estran-17-one) and 19-norepiandrosterone (3 beta-hydroxy-5 alpha-estran-17-one).<sup>5;7;17;59;67;78</sup> These metabolites are isomeric compounds, having the same chemical composition and molecular weight, but different chemical structure. 19-Norandrosterone (19-NA) is usually the most abundant urine metabolite of nandrolone.<sup>5;17;59;67;78</sup> The presence of these metabolites in the urine forms the basis of doping analysis for the illegal use of nandrolone by athletes.<sup>1;7;20;78;91</sup> This was based on the premise that these urine metabolites could only have been derived from exogenous nandrolone. A study in 1982 was suspected measuring 19-NA, or a similar compound, in the urine of individuals who had not used nandrolone.<sup>7</sup> In 1996,

the IOC stated that a critical concentration for nandrolone metabolites in the urine had been established. A doping offence for nandrolone was defined as a concentration of 19-NA in human urine exceeding  $2 \text{ ng}\cdot\text{ml}^{-1}$  in men and  $5 \text{ ng}\cdot\text{ml}^{-1}$  in women.<sup>82</sup>

Recently, questions have been raised regarding possible false positive tests for nandrolone. Explanations for false positive tests have been attributed to a risk of supplement contamination<sup>10</sup> or endogenous production of nandrolone and regulation of metabolic pathways of nandrolone metabolism by various physiological factors and supplement interventions. The aim of this review is to critically analyze the studies on nandrolone metabolism with the overall goal of determining whether it is indeed possible for an athlete to test positive for nandrolone without having either ingested or injected nandrolone. The question of a positive test arising from nutritional supplements<sup>1;10</sup> and food contamination<sup>18;50</sup> is beyond the scope of this review.

## **2.2 Evidence for endogenous 19-norandrosterone**

The origin of endogenous 19-NA found in the urine of athletes not having knowingly ingested or injected nandrolone is central to resolving the question of whether it is possible to have a false positive test. The first study to suggest that 19-NA could be found in the urine of individuals free of exogenous nandrolone was a study on laboratory staff ( $n=14$ ).<sup>7</sup> Their urine was analyzed using isotope dilution-mass spectrometry and 19-NA or a similar compound was suspected. This suspicion was based on the detection of a small peak for the ion at  $m/z$  256.<sup>7</sup> In retrospect, this signal may have been caused by interference of other endogenous compounds (noise) and perhaps represents a false positive finding. The authors acknowledged

the limitations of the study because the analytical technique lacked specificity and sensitivity.

Studies published in 1988 and 1990 again raised the possibility of endogenous 19-NA appearing in the urine of humans. Kicman and Brooks (1988) used radioimmunoassay and measured 19-NA in the urine of men and women, supposedly free of exogenous nandrolone, ranging from 3.8 to 49.4 ng.ml<sup>-1</sup>.<sup>40</sup> However, these data should be interpreted with caution as it could be argued that the analytical technique again lacks both specificity and sensitivity. Debruyckere et al. (1990) measured 19-NA in the urine of three individuals at concentrations of 9, 14 and 37 ng.ml<sup>-1</sup> respectively.<sup>17</sup> These results were later attributed to nandrolone-contaminated meat, which the subjects may have eaten.<sup>18;19</sup>

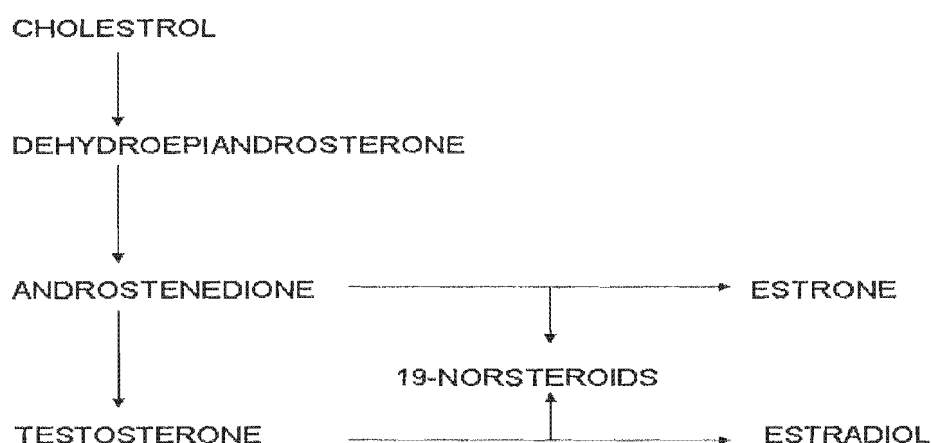
In 1996, the IOC declared that the presence of a small amount of 19-NA in the urine was not considered to constitute a doping offence.<sup>82</sup> This suggests that the authoritative body acknowledged the possibility of endogenous 19-NA production. It can only be assumed that this decision was reached based on the data collected by IOC laboratories during routine drug testing as the scientific evidence at the time was equivocal. In the late 1990's, analytical testing procedures for the detection and quantification of steroid metabolites in urine had become increasingly sensitive.<sup>36</sup> This may have accounted for a significant number of positive urine samples for 19-NA being analyzed in certain anti-doping laboratories. Many of the positive samples were from the participants of sports that had previously not been associated with anabolic steroid use.<sup>51</sup> Further research with more sensitive equipment was undertaken to determine whether NA could be produced naturally by the human body. This research

showed convincing evidence that 19-NA was found in the urine of individuals free of exogenous nandrolone.<sup>10;20;28;28;43;48;51;72;75;79;93</sup> The 19-NA urine concentrations in these studies ranged from 0.01- 1.79 ng.ml<sup>-1</sup>. In a study by Galan Martin et al. (2001), high 19-NA concentrations in five sportspersons (4, 5, 6, 8, 14 ng.ml<sup>-1</sup>) were measured. One woman in the study, who was post-menopausal, had a 19-NA concentration of 22 ng.ml<sup>-1</sup>. It could be argued that these athletes had administered nandrolone.<sup>27</sup> These results are difficult to explain and perhaps further investigation of these subjects is necessary before a definite opinion can be formed.

### 3.3 Metabolism of 19-norandrosterone

#### *Aromatisation*

Metabolic pathways for the endogenous production of 19-NA in the human body need to be considered. Under normal circumstances, testosterone is aromatised to estrogen by the aromatase enzyme complex.<sup>26</sup> Androstenedione, the direct precursor for testosterone, is also aromatised to estrogen by the aromatase enzyme.<sup>29;54</sup> The important step in this metabolic process is the removal of the nineteenth carbon methyl group (CH<sub>3</sub>) from either testosterone or androstenedione. Nandrolone differs structurally from testosterone and androstenedione by lacking the methyl group at the nineteenth carbon position and additionally from androstenedione by substitution of a ketone group for a hydroxyl group at the seventeenth carbon position. Could it be feasible that 19-norsteroids (nandrolone and metabolites) are intermediates in the aromatisation process? (Figure. 2.1).



**Figure 2.1** The androgen biosynthetic pathway and the proposed aromatization of androgen to estrogen with the formation of 19-norsteroid intermediates.

Animal studies, *in vitro* experiments and observations in humans, particularly pregnant females, add support to the proposal that 19-norsteroids are intermediates in the aromatization of androgens to estrogen.<sup>7;10;20;21;40;62;72;73;86;90;93</sup> Estrogen concentrations in women increase significantly both at the time of ovulation and during pregnancy.<sup>57;93</sup> Recently, elevated urine 19-NA levels were identified in women at the time of ovulation<sup>31;93</sup> and during pregnancy.<sup>93</sup> Mareck-Engelke et al. (1998) reported that during pregnancy the concentration of 19-NA in human urine may reach 20 ng.ml<sup>-1</sup>.<sup>58</sup> In these cases, pregnancy is confirmed with a blood test for human chorionic gonadotropin.

A recent study by Reznik et al. (2001), examined the sequelae after the administration of human chorionic gonadotropin to ten male subjects.<sup>72</sup> Human chorionic gonadotropin increases serum testosterone in healthy males<sup>14;41;84</sup> and stimulates the aromatase enzyme causing a gradual increase in serum estrogen.<sup>68;70;84</sup> The serum

testosterone and estrogen increased in the ten subjects after human chorionic gonadotropin administration, and 19-NA excretion in the urine increased by 250%. It may be concluded from this study that the increase in nandrolone biosynthesis was possibly associated with the increased aromatisation of testosterone to estrogen.

Although the pathways proposed are theoretical, the available evidence suggests that it is possible that the flux of androgen precursors down the testosterone biosynthetic pathway could result in the production of endogenous nandrolone. Therefore it can be assumed that factors which could increase the flux of androgen precursors down the testosterone biosynthetic pathway could theoretically increase the amount of nandrolone produced.

## **2.4 Factors with the potential to affect 19-norandrosterone metabolism.**

### *Genetics*

There is a wide range for serum testosterone concentrations in men,<sup>29</sup> suggesting large genetic interindividual and intraindividual variability in sex steroid production and excretion over a 24-hour period.<sup>52</sup> In accordance with this, the possibility exists that there is a variable rate of 19-NA excretion.<sup>51</sup> Indeed, endogenous 19-NA urine excretion in a male athlete varied by 680% over a three-month period.<sup>51</sup> Endogenous 19-NA urine excretion varied in another subject by 72% over a 24-hour period.<sup>20</sup> The enzyme complex, 17 beta-hydroxy-steroid dehydrogenase, responsible for converting androstenedione to testosterone and the aromatase enzyme complex, which converts testosterone to estrogen, occur in muscle and fat.<sup>35;54</sup> Therefore, it is conceivable that individuals with higher muscle and fat content may be more proficient in the

production of 19-norsteroid intermediates. The aromatase enzyme complex *per se* can also show marked genetic variability in expression and activity in certain individuals,<sup>6</sup> with increased activity of the aromatase enzyme producing larger amounts of estrogen. This leads to the question of whether genetic upregulation of the aromatisation process in these individuals increases the production of 19-norsteroids.

### *Exercise*

Intense exercise has been associated with elevated levels of 19-NA in the urine.

<sup>28;48;51;75</sup> Le Bizec et al. (2002) studied professional soccer players over nineteen months and collected 385 urine samples. Urine 19-NA concentrations after soccer games were significantly higher than before games.<sup>48</sup> For 19-NA concentrations after games, 70% of the urine samples were below 0.1 ng.ml<sup>-1</sup>, 20% were between 0.1-0.2 ng.ml<sup>-1</sup>. 19-NA in four urine samples was above 1.0 ng.ml<sup>-1</sup>, the maximum value being 1.79 ng.ml<sup>-1</sup>.<sup>48</sup> A recent study which supported the above hypothesis found that professional football players produced 19-NA in the urine after exercise when compared to sedentary control subjects.<sup>28</sup> However these data should be interpreted with caution because there was no comment in the study on the measurement of baseline urine 19-NA concentrations in the football players before exercise as urine was collected only after exercise. Therefore, it is not possible to comment on the effect of exercise on urine 19-NA concentration. Further, no mention was made about the hydration status of the football players after exercise, particularly as dehydration may have changed the 19-NA concentration in the urine. Also there was no mention on whether the football players were ingesting nutritional supplements, a known

source of possible contamination with norsteroid precursors which may also increase the amount of 19-NA in the urine. <sup>10;16;16;38;38</sup>

When urine is tested for banned substances and the specific gravity of the urine sample is measured above 1.020, urine metabolite concentrations are adjusted by a correction factor. <sup>36</sup> This analysis is based on the premise that urine flow rate and urine metabolite excretion remain constant during and directly after exercise.

However, this is an erroneous assumption as it has been shown that excretion of pseudoephedrine after exercise was increased in subjects in whom urine volume remained constant. <sup>30</sup> Thus, urine metabolite excretion may not remain constant during and directly after exercise and random urine sample collection after exercise may be unreliable. <sup>20;41</sup> A more accurate measure would be to collect a urine sample over a 24-hour period, hence allowing for the calculation of excretion rates of urine metabolites. <sup>20</sup> However, this is not practical, particularly when testing for drug use in sport.

The serum androgen response to exercise in athletes can vary according to the type, duration and intensity of the exercise task. <sup>45;46;55;80;87;94;95</sup> Serum testosterone, androstenedione and dehydroepiandrosterone (DHEA) concentrations increase with short-term, intense exercise due to increased testicular production by an unknown mechanism. <sup>15</sup> An increase in serum testosterone after exercise may also be caused by a decrease in the plasma volume<sup>96</sup> or a decrease in hepatic clearance. <sup>95</sup> The effect of exercise on serum estrogen is also extremely variable. <sup>94</sup>

The hypothesis that exercise may increase the amount of 19-NA in the urine has been challenged in a well controlled laboratory study by Schmitt et al.(2002) who studied male judo players (n=14) and male long distance runners (n=15).<sup>79</sup> Baseline urine samples were collected before a 30 second Wingate exercise test and a treadmill running test to exhaustion and then again at 30 minutes, 60 minutes and 24 hours after exercise. It was found that there was a wide variation in the baseline urine 19-NA concentrations ranging from undetectable to 0.25 ng.ml<sup>-1</sup>. Urine 19-NA levels in the two groups did not differ significantly from pre-exercise values.<sup>79</sup> This study confirms the endogenous production of 19-NA in athletic subjects, however the exercise stress may be considered to be relatively small and therefore not truly representative of longer duration sporting competition stress.

It is conceivable that the increase in circulating androgens in individuals participating in short-duration, high-intensity exercise could result in the stimulation of the aromatase enzyme complex resulting in an absolute increase in the amount of 19-NA appearing in the urine. There are sufficient data<sup>28;48;51;75</sup> to suggest that a urine specimen collected after high-intensity exercise could have a higher concentration of 19-NA for reasons other than dehydration, but this has recently been challenged in a well conducted laboratory study.<sup>79</sup>

#### *Trauma and hypoglycaemic stress*

As yet, no study has investigated the possible effect that traumatic stress (musculoskeletal injury) may have on 19-norsteroid metabolism. Interestingly, two international male athletes, one an international rugby player<sup>64</sup> and the other a ParaOlympian (Personal communication), recently tested positive for 19-NA above 2 ng.ml<sup>-1</sup>, after both had suffered significant injuries just prior to passing a urine sample

for drug testing. Both athletes claimed to be innocent of a doping offence. The concentration of 19-NA in the urine samples of both athletes was approximately 6-8 ng.ml<sup>-1</sup>, which is slightly above the IOC cut-off concentration for men (2 ng.ml<sup>-1</sup>).

Reznik et al. (2001) has provided some insight into the effect of a stress response on nandrolone metabolism.<sup>72</sup> Hypoglycaemia was induced in ten subjects after receiving 0.1 IU.kg<sup>-1</sup> of insulin intravenously. Urine samples were collected at 0-2, 2-4 and 4-10 hours after the insulin injection. They concluded that a hypoglycaemic stress did not significantly alter 19-NA excretion. However, inspection of their data reveals that in certain individuals, 19-NA excretion increased in the first 2 hours after the hypoglycaemic stress was induced. Had the study included more than ten subjects, it is likely there would have been sufficient statistical power to show that the increase in 19-NA in the first 2 hours after a hypoglycaemic stress would have produced a significant finding. Hypoglycaemic stress is associated with the production of glucose counter-regulatory hormones; cortisol, glucagon, growth hormone and adrenaline.<sup>29</sup> Cortisol is produced in the adrenal cortex when stimulated by adrenocorticotrophic hormone (ACTH). ACTH also stimulates the production of androgens and mineralocorticosteroids from the adrenal cortex.<sup>29</sup> It is tempting to speculate that the increased production of adrenal androgens results in increased 19-NA excretion as described above. Further studies need to evaluate whether the increase in adrenal androgens and their aromatisation could produce any changes in 19-NA excretion after a traumatic musculoskeletal stress.

### *Mineral cofactors and herbal products*

There is also a theoretical argument for certain substances, which are not prohibited in sport, altering nandrolone metabolism. For example, the trace element zinc is a cofactor in many enzymatic processes in the body. It has been shown that men, who are marginally zinc deficient, have an increase in serum testosterone following zinc supplementation.<sup>71</sup> Also, diets deficient in zinc resulted in a significant decrease in serum testosterone concentration. Therefore, it can be concluded that zinc supports testosterone production.<sup>71</sup> Although there is a linear relationship between serum zinc and serum testosterone concentrations,<sup>9</sup> it is not known whether supraphysiological doses of zinc are associated with higher levels of testosterone production. Certain athletes are marginally zinc deficient<sup>39</sup> on the basis of inadequate intake<sup>33</sup> and significant sweat losses.<sup>11;13</sup> As zinc status may not be optimal in these athletes, can zinc supplementation enhance testosterone production and could this increase in testosterone production increase the production of aromatisation intermediates? This question was partially addressed when a zinc/magnesium supplement (30 mg zinc) was given to football players nightly for seven weeks. This treatment increased free and bound serum testosterone by approximately 33%.<sup>8</sup> These findings were not attributed to haemoconcentration as the blood samples were taken 24 hours after exercise. Based on the possibility that 19-norsteroid metabolism may be associated with testosterone metabolism and the aromatisation process, it is feasible that zinc supplementation, combined with exercise may increase nandrolone metabolites appearing in the urine.

The herbal product *tribulus terrestris* (tribestan), which has been used in Eastern cultures since ancient times to treat impotence and improve libido, is another substance which has been associated with an increased serum testosterone concentration.<sup>61</sup> Could tribestan in combination with exercise increase NA appearing in the urine? Further research into this question is necessary.

## 2.5 Challenging the IOC cut-off concentration for urine 19-norandrosterone

Until recently, studies involving large numbers of subjects to determine the physiological range for the concentration of 19-NA appearing in the urine of men and women free of exogenous nandrolone were lacking. The available data on the range of 19-NA could only be drawn from the analysis of urine from sedentary and recreational sports persons at rest.<sup>10;20;43;51;72</sup> The total number of subjects from all these studies is approximately one hundred and fifty. No specific mention is made in the studies regarding the age of the male subjects. This is relevant as testosterone production decreases with advancing age.<sup>53</sup> Therefore, one might expect 19-norsteroid production to also decrease with advancing age, making the age of study populations an important consideration. The amount of 19-NA in the urine from the subjects did not exceed  $1 \text{ ng}\cdot\text{ml}^{-1}$ , except in the study of Galan Martin et al. (2001), in which the concerns have already been raised.<sup>27</sup>

Two recent studies involving larger numbers of sportsmen have provided further evidence. Urine samples collected after exercise in these studies showed that the concentration of 19-NA in the urine increased and in certain individuals the concentration of 19-NA was close to the cut-off concentration of  $2 \text{ ng}\cdot\text{ml}^{-1}$ .<sup>48;75</sup> Should

this be combined with other stressors and possible supplement interventions (mentioned above), the concentration of 19-NA in the urine is most unpredictable.

The IOC have also apparently collected data and measured 19-NA urine excretion in elite male and female athletes at the 1996 Nagano Olympic Games. However, these data have not been released into the public domain.<sup>37</sup> It would be beneficial for the IOC data to be made public to support reasoning behind the calculation of cut-off concentrations for 19-NA in the urine of men and women. There is also no reference by the IOC explaining why the threshold concentration for 19-NA is higher in women. If the reason for the 19-NA concentration being higher in women is due to higher circulating levels of estrogen, particularly at the time of ovulation, is this not indirect support for the presence of 19-norsteroids as intermediates in the aromatisation of androgens to estrogen?<sup>93</sup> Bradford-Hill has stated: "It is the essence of science to disclose both the data upon which a conclusion is based and the methods by which the conclusion is obtained".<sup>63</sup>

The IOC has defended the *status quo* on nandrolone and confirmed these threshold values of 2 ng.ml<sup>-1</sup> in men and 5 ng.ml<sup>-1</sup> in women in Monaco in October 1999.<sup>77</sup> The conditions of strict liability are currently applied in the case of any athlete contravening the above thresholds.

## **2.6 Methods to test for 19-norandrosterone**

A solution to the controversy surrounding nandrolone in sport is to develop a testing procedure that can accurately differentiate endogenous nandrolone metabolites from nandrolone that is ingested or injected. The technique of gas chromatography-

combustion-isotope ratio mass spectrometry (GC-C-IRMS) to calculate the  $^{13}\text{C}:^{12}\text{C}$  ratio is currently being developed as a method to fulfil this purpose.<sup>20;51;60;83</sup> This is based on the principle that natural steroids have a different carbon isotopic signature compared to synthetic steroids. The  $^{13}\text{C}:^{12}\text{C}$  ratio for synthetic nandrolone metabolites is lower than the  $^{13}\text{C}:^{12}\text{C}$  ratio for endogenous metabolites, therefore administering exogenous nandrolone will lower this ratio. This ratio has also been proposed as a method of detection for the use of synthetic testosterone as an alternative to the testosterone:epitestosterone ratio.<sup>20;60;83</sup> However, a potential problem with GC-C-IRMS, is the lack of reproducibility and sensitivity due to the low levels of endogenous nandrolone metabolites present in the body. Presently, this method may only be applied to 'high' concentrations of 19-NA ( $60 \text{ ng}\cdot\text{ml}^{-1}$ ) in the urine.<sup>60</sup>

Li Bizec et al. (2002)<sup>49</sup> has proposed examining the steroid conjugates as an additional criterion to distinguish between the endogenous or exogenous origin of nandrolone metabolites. Endogenous 19-NA was found to be 30% sulfo-conjugated as opposed to administered nandrolone, which was found to be 100% conjugated to glucuronic acid when excreted in the urine.<sup>49</sup>

Kintz et al. (2001)<sup>44</sup> proposed that analysis of hair samples from athletes is another option to consider for detecting the presence of exogenous nandrolone. The analysis of hair samples could be used to accurately verify positive results obtained by GC-MS.

42;44

Until the hair sample and GC-C-IRMS techniques have been validated on a large scale, a prudent approach after the detection of 19-NA in urine samples above the cut-

off concentration, is for the athlete to have further blood tests before the sample is declared positive, as is done for athletes with a high testosterone:epitestosterone ratio.

## 2.7 Conclusion

The abuse of the steroid testosterone presented a new problem for drug control in sport.<sup>41</sup> Perhaps the same can now be said for nandrolone. According to the Olympic Movement Anti-Doping Code, 19-NA is not a prohibited substance.<sup>65</sup> However, should 19-NA in the urine exceed a certain threshold concentration, the interpretation is that nandrolone has been ingested or injected. There is strong scientific evidence to show that 19-NA appears in the urine of individuals free of exogenous nandrolone. Evidence suggests that 19-NA may occur as an intermediate in the aromatisation of testosterone to estrogen. Recent evidence has shown that the amount of 19-NA in the urine can be regulated by the administration of human chorionic gonadotropin. Therefore, threshold concentrations for men ( $2 \text{ ng}\cdot\text{ml}^{-1}$ ) and women ( $5 \text{ ng}\cdot\text{ml}^{-1}$ ) as defined by the IOC are still open to debate as conclusive scientific evidence showing how these values may be altered by various physiological stimuli is lacking. In accordance with this, multi-centre studies need to further answer specific questions regarding the current urine threshold concentrations for nandrolone metabolites and whether physiological stressors and permitted supplement interventions can alter 19-NA excretion.

## Chapter 3

### The effect of musculoskeletal injury on endogenous nandrolone

#### 3.1 Abstract

**Background:** Two recent high-profile enquiries about doping infringements, which have involved international male athletes who tested positive for the anabolic steroid nandrolone (19-nortestosterone), have raised the important question of whether severe musculoskeletal injury can affect nandrolone metabolism. Both athletes had sustained severe musculoskeletal injury while exercising and were instructed to pass a urine sample for drug testing. Both athletes denied any deliberate illegal doping practice and had never previously failed a drug test. The concentration of 19-norandrosterone (19-NA) in the urine of both athlete's ranged from 6-8 ng.ml<sup>-1</sup>, slightly higher than the International Olympic Committee (IOC) cut-off concentration of 2 ng.ml<sup>-1</sup> for male athletes. To our knowledge, no study has yet investigated the possible effect that severe musculoskeletal injury may have on the excretion of nandrolone metabolites (19-NA) and 19-noretiocholanolone (19-NE) in the urine of male subjects.

**Purpose:** The main aim of the study was to validate the International Olympic Committee's cut-off concentration (2 ng.ml<sup>-1</sup>) for nandrolone metabolites in the urine of male athlete's immediately after severe musculoskeletal injury.

**Methods:** Descriptive case control study

Thirty two (n=32) healthy male subjects between the age of 18 and 50 years, who had sustained severe musculoskeletal injury, were recruited for the study. Blood and urine samples were collected from the subjects within 3 hours of the injury. Blood samples

were analyzed for plasma cortisol and luteinizing hormone. Urine samples were analyzed for 19-NA and 19-NE and the concentrations reported in  $\text{pg}\cdot\text{ml}^{-1}$ . Baseline urine samples were collected from all subjects six weeks post-injury and analyzed for 19-NA and 19-NE concentrations in the urine.

**Results:** Urine 19-NA did not change significantly post-injury when compared to baseline ( $P = 0.70$ ). 19-NA levels after injury ranged from 2 - 2800  $\text{pg}\cdot\text{ml}^{-1}$  and at baseline ranged from 0 - 540  $\text{pg}\cdot\text{ml}^{-1}$ . Also, urinary 19-NE did not increase significantly post-injury when compared to baseline. Post-injury, 19-NE ranged from 0 - 80  $\text{pg}\cdot\text{ml}^{-1}$  and from 0 - 20  $\text{pg}\cdot\text{ml}^{-1}$  at baseline respectively. Exercise and injury in combination did not significantly change 19-NA or 19-NE when compared to those subjects injured but not exercising. One of the subjects, who had not administered synthetic nandrolone, produced a urine 19-NA value of 2800  $\text{pg}\cdot\text{ml}^{-1}$ , greater than the IOC cut-off concentration of 2000  $\text{pg}\cdot\text{ml}^{-1}$ .

**Conclusion:** This study confirms the endogenous production of small amounts of 19-NA and 19-NE in subjects not administering synthetic nandrolone. Musculoskeletal injury did not result in a significant increase in 19-NA and 19-NE concentrations in the urine when compared to baseline. One of the injured subjects, free of synthetic nandrolone, produced a 19-NA concentration of 2800  $\text{pg}\cdot\text{ml}^{-1}$  (IOC cut-off = 2000  $\text{pg}\cdot\text{ml}^{-1}$ ). This suggests that at least 1 in 32 subjects who have a severe musculoskeletal injury will exceed the IOC cut-off concentration for 19-NA (2000  $\text{pg}\cdot\text{ml}^{-1}$ ). Further research needs to examine the mechanisms causing an increase in 19-NA after musculoskeletal injury so that the IOC guidelines for a positive nandrolone drug test can be adjusted to reduce the risk of an athlete having a false positive test.

Furthermore, ethical issues with regards to performing drug testing on athletes after sustaining severe musculoskeletal injury needs to be considered.

**Key words:** 19-NORANDROSTERONE, 19-NORETIOCHOLANOLONE, MUSCULOSKELETAL INJURY, ADRENAL ANDROGENS, AROMATISATION, INJURY SEVERITY SCORE.

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### 3.2 Introduction

Nandrolone (19-nortestosterone) is an anabolic androgenic steroid currently banned by the International Olympic Committee (IOC).<sup>65</sup> Exogenous nandrolone administration in athletes is detected by analyzing the urine for the three metabolites of nandrolone namely 19-norandrosterone (19-NA), 19-noretiocholanolone (19-NE) and 19-norepiandrosterone (19-NEA).<sup>5;7;17;59;67;78</sup> The detection of 19-NA above a urine concentration of 2 ng.ml<sup>-1</sup> in male athletes and 5 ng.ml<sup>-1</sup> in non-pregnant female athletes is regarded as evidence of exogenous nandrolone administration and constitutes a doping offence according to IOC doping regulations.<sup>65;67</sup>

Nandrolone in healthy men is undetectable in the plasma (< 4 pg.ml<sup>-1</sup>),<sup>5;73</sup> however, recent scientific evidence has confirmed that small amounts of nandrolone metabolites can be detected in the urine of humans who have not administered exogenous nandrolone.<sup>10;20;28;43;48;51;72;75;79;93</sup> Therefore, it may be concluded that small amounts of nandrolone are produced endogenously in the human body. The precise endocrine pathway for endogenous nandrolone production has yet to be established. However, animal studies, *in vitro* experiments and research from humans, particularly pregnant woman, add support to the proposal that 19-norsteroids are intermediates in the aromatisation of androgens to estrogen.<sup>7;10;20;21;40;62;72;73;86;90;93</sup> This metabolic pathway has the potential to be upregulated, as demonstrated by the increase in the concentration of 19-NA in the urine of humans after the administration of human chorionic gonadotropin.<sup>72</sup> This important finding raises the possibility that other factors may alter the production and excretion of nandrolone metabolites in the urine of humans.

Anecdotal evidence presented at two recent doping infringement enquiries raised the possibility of an interaction between musculoskeletal injury and the concentration of 19-NA in the urine of humans. Two international male athletes tested positive for nandrolone when the concentration of 19-NA in their urine exceeded  $2 \text{ ng}\cdot\text{ml}^{-1}$ . Both athletes had sustained significant musculoskeletal injuries immediately prior to passing a urine sample for drug testing. One athlete was an international rugby player, who sustained a wrist bone fracture<sup>64</sup>. The other athlete was a ParaOlympian wheelchair sprinter (Personal communication), who dislocated his shoulder while overreaching for the finish line. The concentration of 19-NA in the urine samples of both athletes was approximately  $6\text{-}8 \text{ ng}\cdot\text{ml}^{-1}$ , which is slightly above the IOC cut-off concentration for men ( $2 \text{ ng}\cdot\text{ml}^{-1}$ ). Both athletes denied having ever used any nandrolone containing products previously.

To date, no study has investigated the possible effect of musculoskeletal injury on the excretion of nandrolone metabolites in the urine of male subjects. Accordingly, the aim of this study was to determine if musculoskeletal injury in male subjects could increase the concentration of nandrolone metabolites (19-NA and 19-NE) in the urine.

### **3.3 Methods**

#### *Study design*

A case-control study was conducted in the Accident and Emergency Unit of Milnerton Medi-Clinic, South Africa, following ethical approval by the Ethics and Research Committee of the Faculty of Health Sciences, University of Cape Town, South Africa.

### Subjects

Thirty two (n=32) male subjects admitted to the Milnerton Medi-Clinic Accident and Emergency Unit with severe musculoskeletal injuries were recruited for the study. The exclusion criteria were strictly applied (Appendix D). The subjects were all physically active, competing on a recreational level and denied any current or previous anabolic steroid use. The descriptive characteristics of the subjects are shown in Table 3.1. The average age of the subjects was  $30.8 \pm 7.6$  years (range 18-43 years). The average values for body mass, stature and body mass index are  $82.3 \pm 16.6$  kg,  $176.1 \pm 16.6$  cm and  $26.5 \pm 5.0$  kg.m<sup>-2</sup> respectively. All subjects performed regular weekly exercise activity, training on average  $242 \pm 161$  min.week<sup>-1</sup> (range 60 – 720 min.week<sup>-1</sup>) and fulfilled the inclusion criteria for being moderately active individuals.

**Table 3.1** Descriptive characteristics of the subjects (n=32).

N = 32	Mean $\pm$ SD	Minimum	Maximum
Age (years)	$30.8 \pm 7.6$	18.0	43.0
Body mass (kg)	$82.3 \pm 16.6$	50.0	125.0
Stature (cm)	$176.1 \pm 16.6$	154.0	196.0
BMI (kg.m <sup>-2</sup> )	$26.5 \pm 5.0$	19.8	41.3
Training time (min.wk <sup>-1</sup> )	$242 \pm 161$	60	720

All subjects required treatment for a musculoskeletal injury and presented to the emergency unit for treatment within 3 hours of sustaining the injury. A medical history and examination were performed. If a bone fracture, joint dislocation or tendon rupture was suspected clinically and less than 3 hours had elapsed, the subject was asked to participate in the study. Subjects gave written informed consent after the study protocol had been fully explained to them. The time of the injury was

documented. A blood sample to measure plasma cortisol and luteinizing hormone and a urine sample to measure 19-NA and 19-NE concentrations were collected from all the subjects. The time of blood and urine sample collection was documented. Analgesia was administered after the collection of urine and blood samples.<sup>85</sup> X-rays were performed to confirm the diagnosis. After the X-ray, subjects were assigned an Injury Severity Score (ISS).<sup>2</sup> To calculate the ISS, the injury was graded as minor =1, moderate =2, severe but not life threatening =3, severe life threatening =4 or critical =5. If one injury was present, the score that was allocated for that injury is squared. If two injuries were present, the score for each injury was squared and both scores added together.<sup>2</sup> Based on the criteria for this study, an ISS for a single bone fracture, joint dislocation or tendon rupture ranged from an ISS score of 4-9. Subjects with an ISS of 4-13 were eligible for the study. Subjects completed questionnaires to document demographic details and a training history. All subjects received standard appropriate medical treatment for their respective injury. The treatment was not altered by their participation in the study. Six weeks after the injury, a baseline urine sample was collected from the subjects (n=32) for the measurement of 19-NA and 19-NE concentrations. No blood samples were collected six weeks after the injury.

#### *Blood sampling and analytical procedure*

Blood samples (5 ml) were collected using strict aseptic technique from the right antecubital vein of each of the subjects (n=32) and placed into SST® Gel and Clot Activator vacutainers (Preanalytical Systems, Plymouth, UK). Immediately after collection, the blood was centrifuged at  $3000 \times g$  for ten minutes. Plasma cortisol and luteinizing hormone (LH) were assayed using a competitive immunoassay with chemiluminescent technology (Advia Centaur, Bayer Corporation, Tarrytown, NY,

USA). Plasma cortisol was quantified in  $\text{nmol.L}^{-1}$  and LH results were quantified in  $\text{IU.L}^{-1}$ .

#### *Urine sample collection and transportation*

Urine samples were collected within 3 hours of sustaining a musculoskeletal injury. Urine specific gravity (SG), urine pH and urine creatinine were measured as approximate indicators of the subject's hydration status. Urine aliquots (40 ml) were stored at  $-20\text{ }^{\circ}\text{C}$ . Although it is recommended that urine be stored under these conditions, a recent research study has shown that different storage conditions do not significantly alter the concentration of nandrolone metabolites in the urine.<sup>92</sup> The samples were couriered by DHL Express to Hall Analytical Laboratories in Manchester, United Kingdom. The cold chain was maintained throughout the transportation.

#### *Urine 19-NA and 19-NE steroid analysis*

At Hall Analytical Laboratories (Manchester, United Kingdom), all samples were stored in a freezer at  $-80\text{ }^{\circ}\text{C}$ . Before analysis, each sample was removed from the freezer and allowed to thaw at room temperature for 60 minutes. Ten ml of urine were decanted from each sample and placed into individual 50 ml glass sample vials. Any excess urine was returned to the freezer ( $-80\text{ }^{\circ}\text{C}$ ).

#### *Steroid extraction and purification*

Before steroid extraction and purification, 10 ng of testosterone- $\text{d}_3$  (Sigma standard T-5536, Steinheim, Germany) was added to the urine as an internal standard. The steroid content of the sample was hydrolyzed with 0.5 ml (minimum 50 IU) of E. Coli  $\beta$ -

glucuronidase (Sigma g-8396) for 60 minutes at 52 °C (buffered at pH 6.8). The hydrolyzed sample was applied to a pre-conditioned C-18 cartridge (Supelco DSC-18 6 ml tubes, 52606-U, Sigma-Aldrich Co. 2001, Bellefonte, USA), which was then flushed with 6 ml of de-ionized water and 6 ml of hexane. The sample was then eluted from the column by flushing with 6 ml of methanol. Once eluted, the extract was dried under a constant flow of nitrogen at a temperature of 40 °C.

The dried residue was diluted in 500 µl of hexane / dichloromethane (60:40 v/v) and placed in an ultrasonic bath for 30 minutes. The extract was then applied to a preconditioned DSC-Si silica tube (Supelco DSC-Si 6 ml tubes, 52656-U, Sigma-Aldrich Co. 2001, Bellefonte, USA). This procedure was carried out under low flow rates with 10 ml of hexane. Following sample application, the column was allowed to dry for 3 minutes and then flushed with 8 ml of hexane / ethylacetate (85:15 v/v). The sample was eluted with 13 ml of hexane and ethylacetate (60:40 v/v) and the resultant solution blown to dryness under a constant stream of nitrogen at 40 °C.

#### *Derivatization*

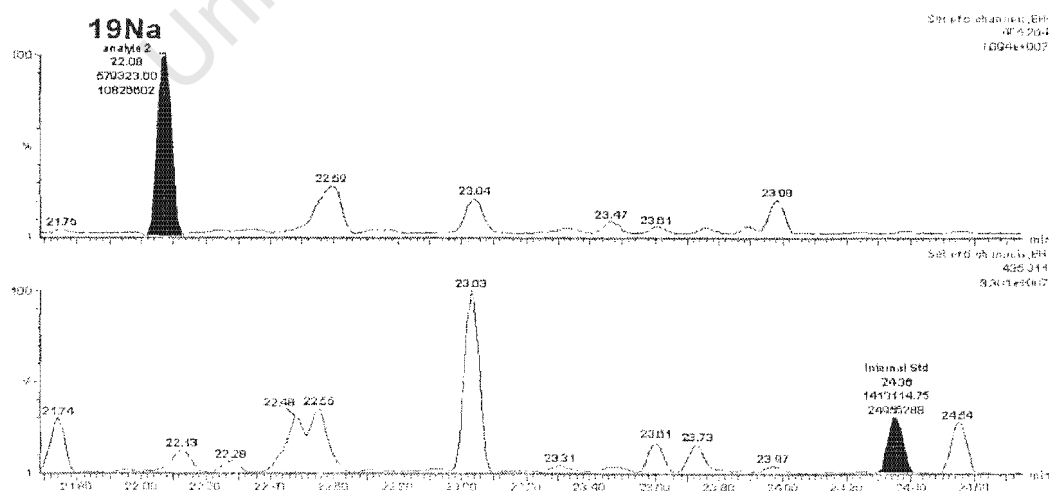
The dry residue was derivatized with N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA, Sigma 39,486-6, ) and N-Trimethylsilylimidazole (TMSI, Sigma 39,487-4 Steinheim, Germany) (100:3 v/v) at 50 °C for 120 minutes to form Trimethylsilyl-enol-trimethylsilyl - ethers.

#### *GC-MS analysis*

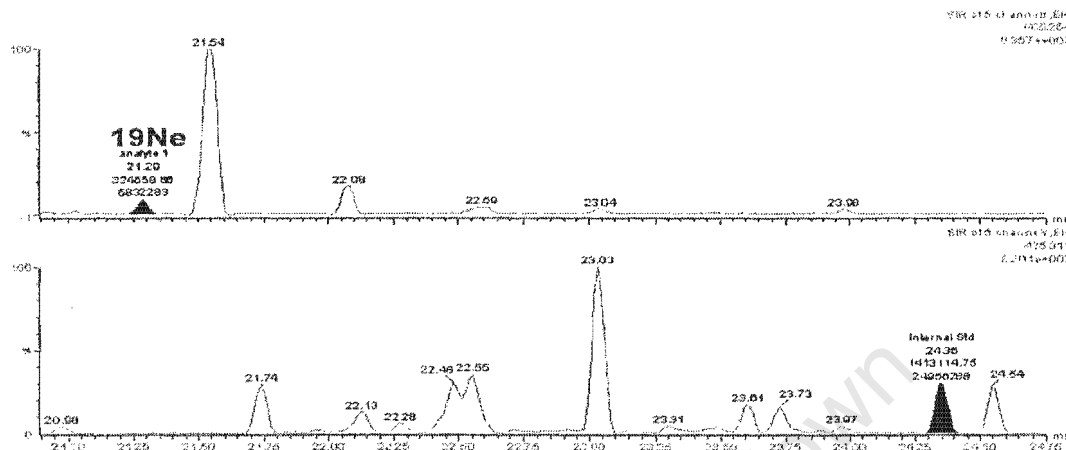
Analysis was performed on a Micromass Autospec high-resolution mass spectrometer (Micromass UK Ltd, Manchester, UK) coupled to a Hewlett-Packard 5890 Gas Chromatograph (Palo Alto, CA, USA). A DB-5 MS 30 m column, with a film thickness

of 0.25  $\mu\text{m}$ , was used to achieve chromatographic separation. The GC was run in splitless mode, with an injection temperature of 280  $^{\circ}\text{C}$  and a constant pressure of 70 kPa. The temperature of the oven was set initially to 80  $^{\circ}\text{C}$  for 2 minutes, and increased at 10  $^{\circ}\text{C}$  per minute to 200  $^{\circ}\text{C}$ . The rate of temperature increase was then decreased to 4  $^{\circ}\text{C}$  per minute until a final temperature of 315  $^{\circ}\text{C}$  was reached, the GC oven was then held constant at this temperature for a further 15 minutes.

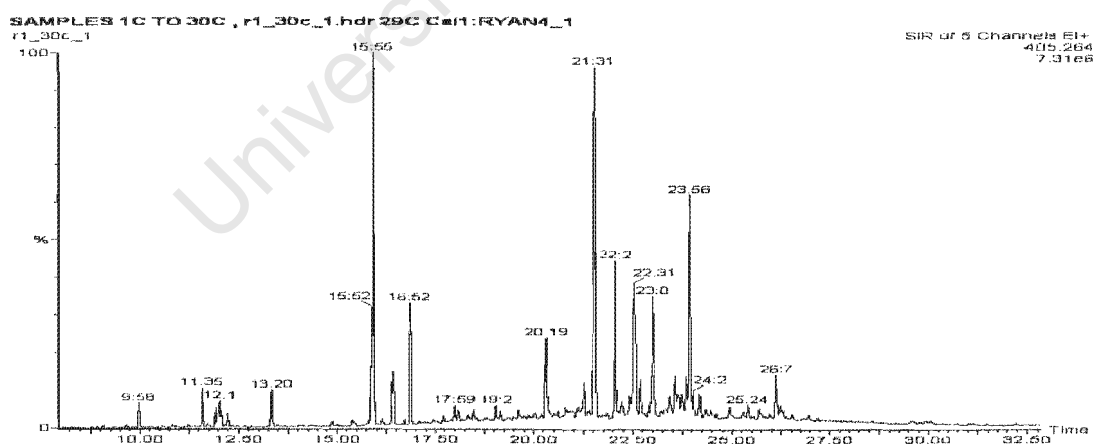
The mass spectrometer was run at a resolution of 5000 Hz (10% valley definition) whilst monitoring ions with an  $m/z$  ratio of 435, 420 and 405. To improve the resolution of the system the less abundant ions ( $m/z$  215 and 315) were not monitored although their presence had been determined prior to high-resolution analysis. Heptacosane ( $m/z$  414) was used as the lock mass. A 1  $\mu\text{l}$  injection of sample proved sufficient for peak detection and quantification to produce above average chromatographic and mass spectrum data for unambiguous identification of 19-NA and 19-NE. A typical spectrograph mass chromatogram obtained by this method can be seen in Figure 3.1 a, b, c.



**Figure 3.1a** Single ion monitoring spectrograph mass chromatogram ( $m/z$  405) of the 19-NA and 19-NE content of an athlete's urine following acute musculoskeletal injury.



**Figure 3.1b** Single ion monitoring mass chromatogram ( $m/z$  405) of the 19-NE content of an athlete's urine following acute musculoskeletal injury.



**Figure 3.1c.** Single ion monitoring spectrograph ( $m/z$  405) of the 19-NA and 19-NE content of an athlete's urine following acute musculoskeletal injury.

### *Quantification*

Calibration was carried out using 19-NA and 19-NE standards purchased from Steraloids (Wilton, NH, USA). Following the injection of 10 ng of testosterone-d<sub>3</sub>, the standards were derivatized and analyzed in the same manner as the samples (as described above). The calibration curve consisted of seven points distributed from 1 to 5000 pg. ml<sup>-1</sup> producing a linear line of best fit with an  $r^2$  of  $\geq 0.98$ . The detection limit of the Autospec was  $<10$  pg.ml<sup>-1</sup> for 19-NA and 19-NE. As such 1 pg. ml<sup>-1</sup> was taken as the limit of accurate analysis. Analytical recovery was greater than 90 %. The 19-NA and 19-NE concentrations are expressed in pg.ml<sup>-1</sup>. To convert to the units reported by the IOC (ng.ml<sup>-1</sup>), divide pg.ml<sup>-1</sup> by 1000.

### *Statistical analysis*

All values are presented as the mean  $\pm$  standard deviation (mean  $\pm$  SD). Statistical analyses were performed using the Statistica software version 6.1 (Statsoft, Inc, Tulsa, OK, USA). Data were analysed using the dependent  $t$  test to compare samples collected at post-injury and at baseline. Where data showed significant variance and skewness in distribution and did not fulfill the criteria for normally distributed parametric data, the Wilcoxon Matched Pairs test was used to determine differences. Pearson's Product Moment Correlation was used to establish the relationship between variables. A Spearman's Rank Order Correlation was used to establish a relationship between the ISS score and the other variables. Statistical significance was accepted as  $P < 0.05$ .

### 3.4 Results

#### *Injury characteristics of the subjects*

The injury characteristics of the subjects are shown in Table 3.2. Twenty six subjects sustained a bone fracture (81 %), four subjects sustained a joint dislocation (13 %) and two subjects sustained an Achilles tendon rupture (6 %). The injury severity score ranged from 4-12 arbitrary units, fulfilling the criteria in the study for a moderate to severe injury.

**Table 3.2** Descriptive characteristics of the injuries (n=32).

	Injury type 1 <sup>#</sup>	Injury type 2 <sup>*</sup>	Injury type 3 <sup>φ</sup>
Number of subjects	26 (81 %)	4 (13 %)	2 (6 %)
Injury severity score (arbitrary units)	9 ± 3	9 ± 0	9 ± 0

<sup>#</sup> injury type 1 = bone fracture <sup>\*</sup> injury type 2 = joint dislocation <sup>φ</sup> injury type 3 = tendon rupture

The data describing the time of the musculoskeletal injury and the time of urine sample collection are shown in Table 3.3. The majority of injuries occurred just after midday (13h 28 min ± 3h 04 min), with the earliest injury occurring at 04h 00 min in the morning and the latest injury occurring at 18h 00 min in the evening. All urine samples were collected within the prescribed 3 hour cut-off period after injury (1h 16 min ± 0h 40 min) as defined for the study protocol. The earliest urine sample was collected within 25 minutes of the injury occurring and the latest urine sample was collected 3 hours after the injury.

**Table 3.3** The time of the musculoskeletal injury and urine sample collection in the subjects.

	Mean $\pm$ SD	Minimum	Maximum
Time of injury (h:min)	13h 28 min $\pm$ 3h 04 min	04h 00 min	18h 00 min
Time of urine collection	14h 44 min $\pm$ 2h 58 min	07h 00 min	19h 19 min
$\Delta$ time between injury and urine collection	1h 16 min $\pm$ 0h 40 min	0h 25 min	3h 00 min

#### *Plasma hormone concentrations*

The plasma hormone concentrations of the subjects post-injury are shown in Table 3.4. The average plasma cortisol was  $739 \pm 316$  nmol.L<sup>-1</sup> and varied widely from 326 to 1564 nmol.L<sup>-1</sup> post-injury (Figure 3.2). The plasma cortisol values represent a significant stress response to the musculoskeletal injury compared to basal diurnal plasma cortisol concentrations reported as ranging from 85 to 620 nmol.L<sup>-1</sup> (Pathcare Laboratories, South Africa) (Figure 3.3). All the plasma LH concentrations were within the normal physiological range (1.0 – 15.0 IU.L<sup>-1</sup>)(Pathcare Laboratories, South Africa). The average plasma LH was  $5.6 \pm 3.4$  IU.L<sup>-1</sup> and varied widely from 1.1 to 12.7 IU.L<sup>-1</sup> (Figure 3.4).

**Table 3.4** Plasma cortisol and LH responses in male subjects post-injury (n=32).

	Mean $\pm$ SD	Minimum	Maximum
Plasma cortisol (nmol.L <sup>-1</sup> )	$739 \pm 316$	326	1564
Plasma LH (IU.L <sup>-1</sup> )	$5.6 \pm 3.4$	1.1	12.7

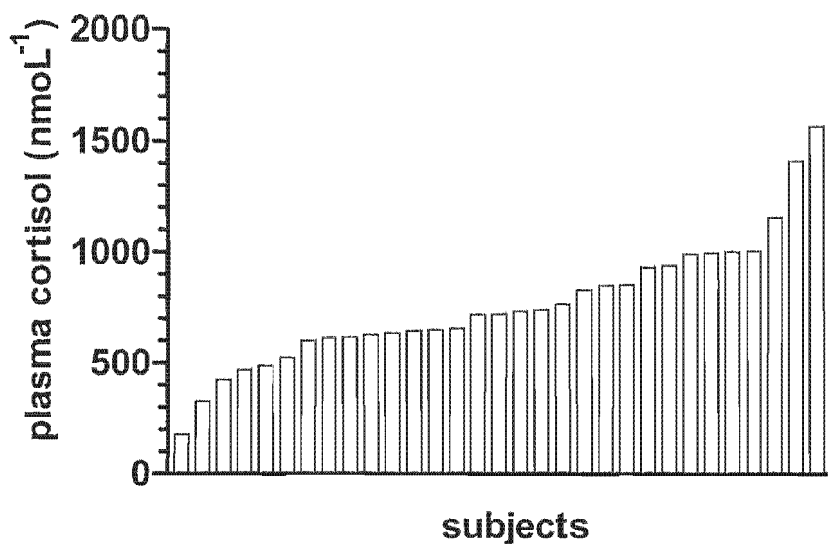


Figure 3.2 Inter-individual variation in plasma cortisol concentrations post-injury.

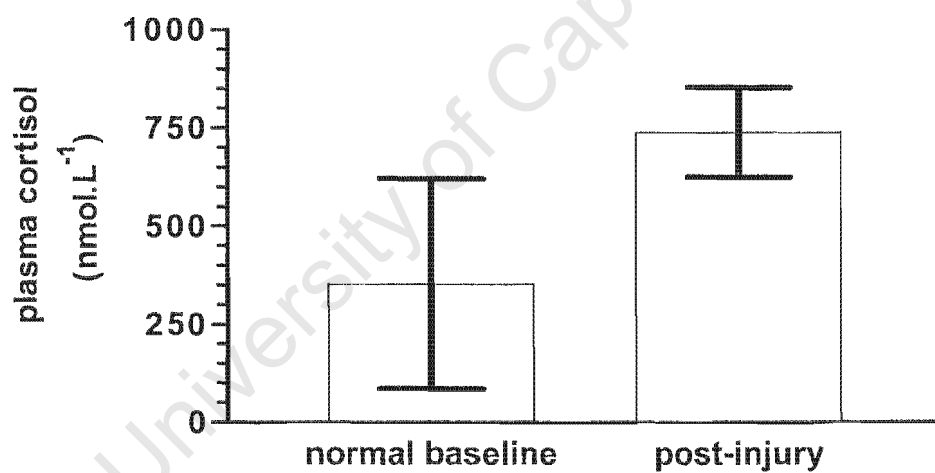
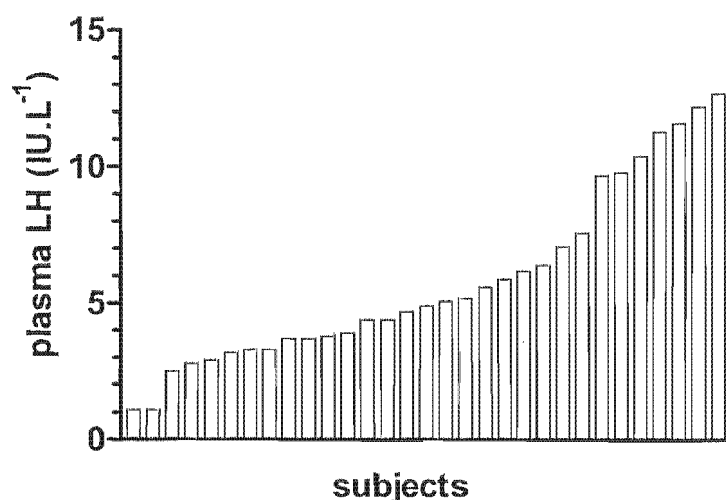


Figure 3.3 Plasma cortisol concentrations (mean  $\pm$  SD) post-injury are compared to normal baseline.



**Figure 3.4** Inter-individual variation in plasma LH concentrations post-injury.

#### *Urinary characteristics*

The urine characteristics of the subjects are shown in Table 3.5. Urine creatinine, SG and pH were all within the normal range and therefore unlikely to affect the analysis of the urine samples for nandrolone metabolite concentrations.

**Table 3.5** Urine characteristics in male subjects after sustaining musculoskeletal injury (n=32).

Variable	Mean $\pm$ SD	Minimum	Maximum
Urine creatinine (mmol.L <sup>-1</sup> )	19.7 $\pm$ 8.1	4.5	36.1
Urine SG	1.024 $\pm$ 0.007	1.010	1.030
Urine pH	5.9 $\pm$ 0.6	5.0	7.5

#### *Urinary 19-NA and 19-NE concentrations*

The urine 19-NA and 19-NE concentrations of the subjects post-injury and at baseline are shown in Table 3.6. 19-NA at baseline was 140  $\pm$  130 pg.ml<sup>-1</sup> and ranged from

undetectable levels to 540 pg.ml<sup>-1</sup> (Figure 3.5 (a)). 19-NA post-injury averaged 169 ± 488 pg.ml<sup>-1</sup>. There was a wide individual variation ranging from 2 - 2800 pg.ml<sup>-1</sup> (Figure 3.5 (b)). There was no significant difference between 19-NA in the urine post-injury when compared to baseline (P = 0.7) (Figure 3.6(a)). There was a significant increase in 19-NE post-injury compared to baseline (P < 0.05)(Figure 3.6(b)). 19-NE at baseline averaged 6 ± 5 pg.ml<sup>-1</sup>, ranging from undetectable levels to 20 pg.ml<sup>-1</sup> (Figure 3.7(a)). 19-NE post-injury was 13 ± 19 pg.ml<sup>-1</sup>, ranging from undetectable levels to 80 pg.ml<sup>-1</sup> (Figure 3.7(b)). If the subject with the high level of 19-NA and 19-NE is excluded on the basis of being an outlier, then the difference in 19-NE was not significant (P = 0.08 dependant *t* test). Similarly, if the data are analyzed with a Wilcoxon Matched Pairs test for non-parametric data, the 19-NE values are not significantly different (post-injury vs baseline)(P = 0.1)

**Table 3.6** Urinary 19-NA and 19-NE concentrations immediately post-injury and at baseline.

	Post-injury	Baseline
Urine 19-NA (pg.ml <sup>-1</sup> )	169 ± 488	140 ± 130
Urine 19-NE (pg.ml <sup>-1</sup> )	13 ± 19	6 ± 5

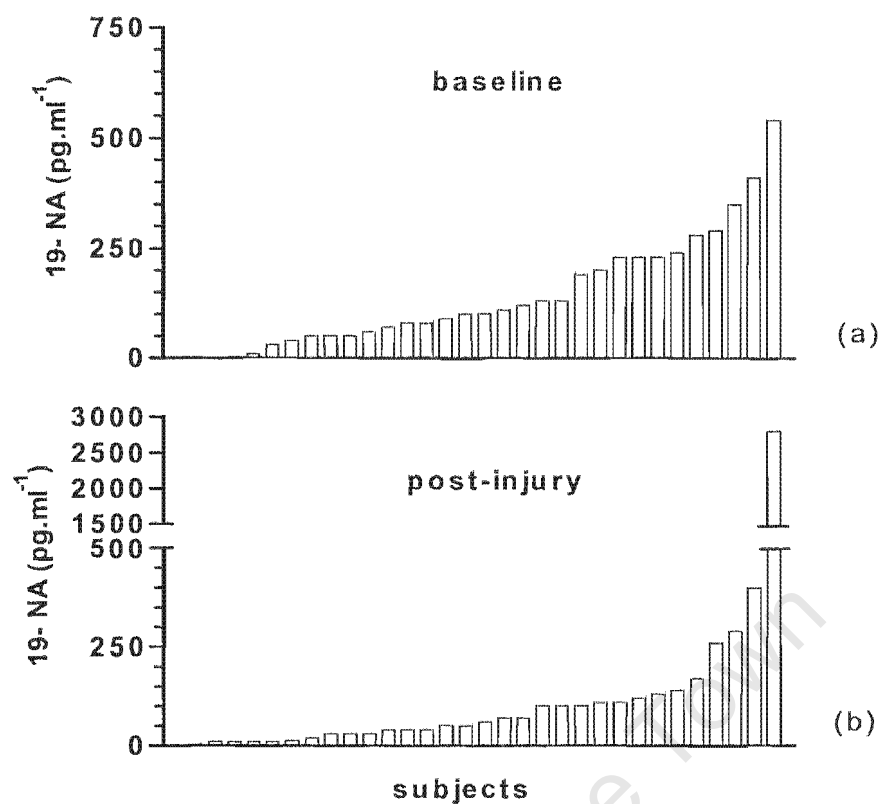
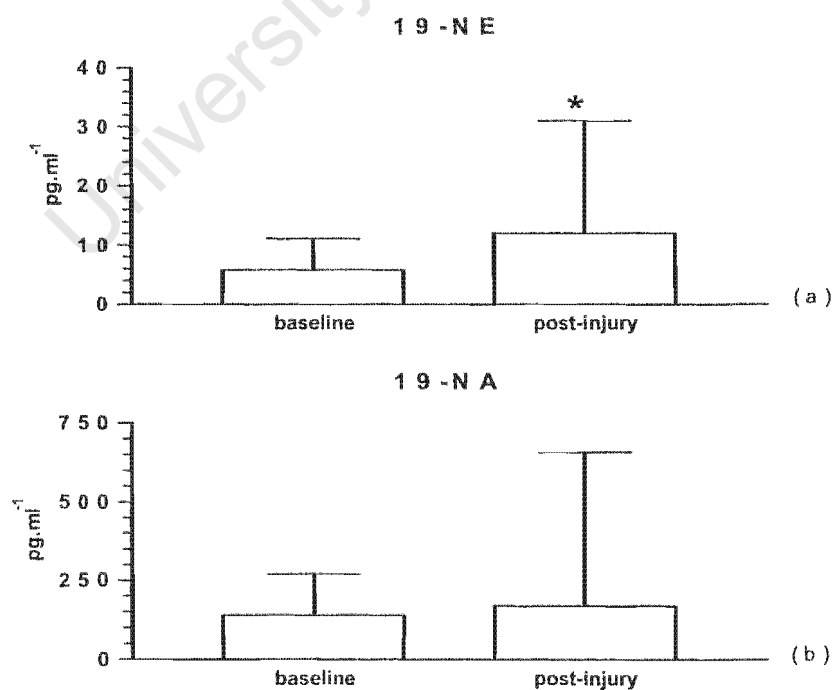
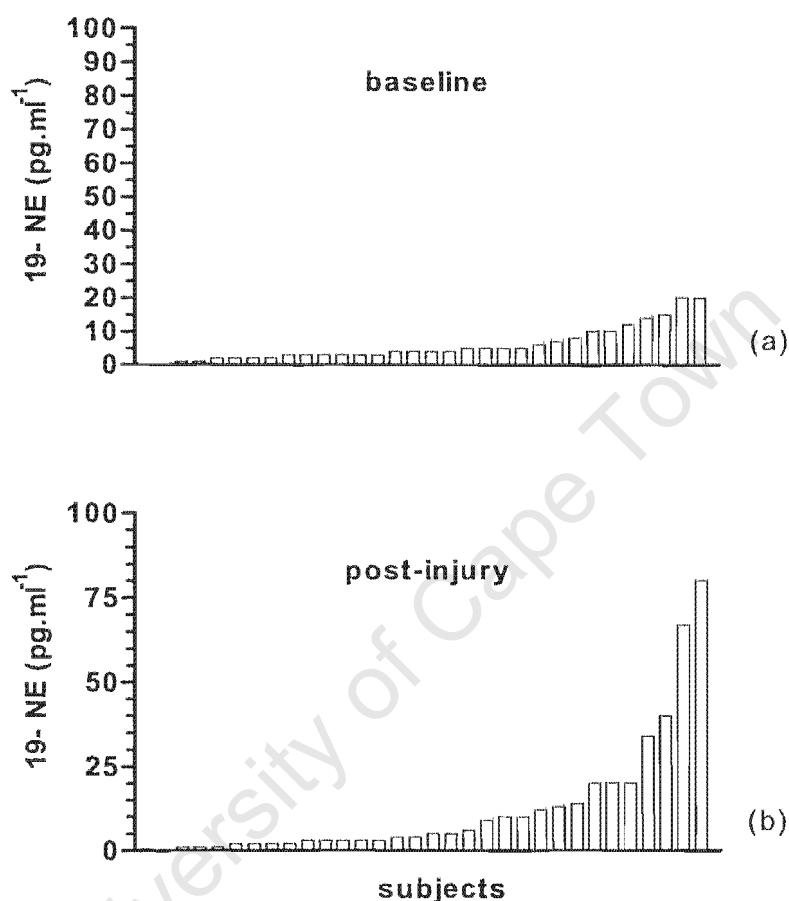


Figure 3.5 (a) and (b) Inter-individual variation in urinary 19-NA concentrations at baseline (a) and post-injury (b).



**Figure 3.6 (a) and (b)** Urine 19-NE ( $\text{pg}\cdot\text{ml}^{-1}$ ) post-injury and at baseline (a) and urine 19-NA ( $\text{pg}\cdot\text{ml}^{-1}$ ) post-injury and at baseline (b)( $n=32$ ).  
\*  $P < 0.05$  (all data included).



**Figure 3.7 (a) and (b)** Inter-individual variation in urinary 19-NE concentrations at baseline (a) and post-injury (b).

There was no significant relationship between the body mass index of the subjects and urinary 19-NA ( $P = 0.59$ ) and 19-NE ( $P = 0.29$ ) post-injury. Also, there was no significant relationship between plasma cortisol post-injury and ISS ( $P = 0.36$ ). Urine pH, urine SG and urine creatinine were not significantly related to urinary 19-NA and

19-NE both post-injury and at baseline. Further, training time per week was not related to urinary 19-NA ( $P = 0.73$ ) and 19-NE ( $P = 0.63$ ) after the injury.

The subjects in the study were further subdivided into 2 sub-groups. Sub-group A sustained musculoskeletal injury while performing exercise ( $n=20$ ) and Sub-group B sustained musculoskeletal injury while at rest ( $n=12$ ) (Table 3.7). Both groups showed a similar increase in cortisol concentrations post-injury and hence showed a similar stress response, with no significant difference between the sub-groups ( $743.8 \pm 326.3$  vs  $731.7 \pm 313.0$ ; exercise vs rest;  $P = 0.09$ ). There was no significant difference between subgroups in urinary 19-NA post-injury ( $P = 0.28$ ) and at baseline ( $P = 0.86$ ). Similarly, there was no significant difference in urinary 19-NE post-injury ( $P = 0.43$ ) and at baseline ( $P = 0.47$ ) between the sub-groups.

**Table 3.7** Urinary 19-NA and 19-NE concentrations for Sub-group A ( $n=20$ ) and Sub-group B ( $n=12$ ).

	post-injury		baseline	
	19-NA ( $\text{pg.ml}^{-1}$ )	19-NE ( $\text{pg.ml}^{-1}$ )	19-NA ( $\text{pg.ml}^{-1}$ )	19-NE ( $\text{pg.ml}^{-1}$ )
Sub-group A	$95.9 \pm 98.1$	$14.6 \pm 22.5$	$137.2 \pm 129.5$	$6.4 \pm 5.9$
Sub-group B	$291.7 \pm 793.4$	$9.0 \pm 9.6$	$145.8 \pm 137.2$	$4.9 \pm 4.1$
P-value	0.28	0.43	0.86	0.47

Sub-group A: Performing exercise when sustaining musculoskeletal injury; Sub-group B: At rest when sustaining musculoskeletal injury.

### 3.5 Discussion

The main finding in this study was that one of the subjects, who had not reportedly administered exogenous nandrolone and who did not consume any nutritional supplements, produced a urine 19-NA concentration post-injury which exceeded the IOC cut-off concentration of  $2000 \text{ pg}\cdot\text{ml}^{-1}$ <sup>65,67</sup>. This finding represents a false positive result for a nandrolone drug test. This may represent a one in thirty two chance of an individual producing 19-NA in the urine above the IOC cut-off concentration after severe musculoskeletal injury. It is not known how often athletes who have sustained severe musculoskeletal injury are instructed to pass urine for drug testing, however this finding would suggest that a false positive finding after sustaining musculoskeletal injury may not be an uncommon finding. There was a wide variation in the 19-NA concentration in the urine of the subjects and this value ( $2800 \text{ pg}\cdot\text{ml}^{-1}$ ) does represent a significant outlier. The result warrants a comprehensive endocrinological investigation and the simulation of a further stress response to determine if this result is reproducible in this subject.

Secondly, musculoskeletal injury did not consistently increase the concentration of 19-NA in the urine of subjects post-injury when compared to baseline. There was a tendency for the urine concentration of 19-NE to increase post-injury when compared to baseline. However, the data lacked statistical power making it difficult to show significant difference. The data were biased as one of the subjects had unusually elevated 19-NA and 19-NE concentrations post-injury. If this subject was excluded from the analysis on the basis of being an outlier, then it becomes apparent that the concentration of 19-NA in the urine tends to be lower post-injury when compared to baseline, although not statistically significant.

This is the first study in humans which has investigated the possible effect that a severe physiological stress may have on the excretion of nandrolone metabolites (19-NA and 19-NE) in the urine. The question became apparent after two elite male athletes tested positive for nandrolone after sustaining severe musculoskeletal injury.<sup>{41}</sup> Also, Reznik et al.<sup>72</sup> has shown that the excretion of 19-NA in the urine may show an upward trend within 2 hours of a physiological hypoglycaemic stress response. This finding was however not significant, possibly because of the low statistical power as a result of the small sample size (n=10).<sup>72</sup>

All subjects in the present study had normal plasma LH concentrations, indicative of normal function of the hypothalamic-pituitary axis. This suggests indirectly that exogenous androgenic anabolic steroids had not been administered as androgenic anabolic steroids may suppress the hypothalamic-pituitary axis and attenuate LH secretion.<sup>56;88</sup> Additionally, plasma cortisol concentrations increased significantly post-injury, in accordance with a physiological stress response.<sup>81</sup> The rapid increase in cortisol is mediated by ACTH which increases immediately after injury.<sup>81</sup> Within 2 hours of moderate to severe injury (ISS < 13), plasma cortisol and ACTH increase in parallel.<sup>4</sup> Therefore, blood samples were collected within 3 hours of injury in an attempt to ensure that the adrenal cortex was being maximally stimulated by ACTH. All subjects were not shocked and perfusing all organs normally. Plasma cortisol measured after injury is not influenced by diurnal variation<sup>4</sup> and therefore the timing of blood sampling did not have to be standardized in this study. ACTH acts as the sole stimulus of the adrenal cortex resulting in the excretion of adrenal androgens, glucocorticoids and mineralocorticoids.<sup>4;29</sup> In planning this study it was hypothesized that during a physiological stress the adrenal cortex could be a source of adrenal

androgens and therefore increase the excretion of aromatisation intermediates. In this study, the adrenal cortex would have been maximally stimulated post-injury and if adrenal androgens were increased, then it is feasible that they would have increased the excretion of 19-NA and 19-NE. There is evidence to show that the dog adrenal gland has the aromatase enzyme <sup>66</sup>. However, in porcine and human adrenal glands, the aromatase enzyme complex may not be present <sup>12;97</sup> Therefore it is unlikely that any increase in adrenal androgens would be aromatized, possibly explaining why 19-NA and 19-NE did not increase consistently in this study. The findings of our study would seem to support the above, although adrenal androgens were not measured.

In healthy individuals, testicular androgens have been shown to decrease during a physiological stress response <sup>24;72</sup> due to cortisol binding to Leydig cell receptors, down-regulation of LH receptors or a centrally-mediated inhibition of LH secretion. <sup>3;25;69;74</sup> The decrease in the androgen concentrations could therefore result in a decrease in the urinary excretion of 19-NA and 19-NE. This did not occur so the proposed mechanism needs to be re-evaluated together with the measurement of testicular androgens. Therefore, one may have expected a similar response in the subjects in this study post-injury, however testicular androgens were not measured in this study.

Another important finding in this study is the confirmation of the endogenous production of small amounts of nandrolone metabolites in the urine post-injury and at baseline (Table 3.6). This is in agreement with the findings of various other studies in humans. <sup>10;20;28;28;43;48;51;72;75;79;93</sup> The above findings are also consistent with other studies which have found that 19-NA is the most abundant metabolite of nandrolone

and is excreted in the urine in higher concentrations compared to 19-NE.<sup>5;17;59;67;78</sup> (Table 3.6) 19-NE concentrations were undetectable in the urine of some subjects, which is also consistent with other study findings.<sup>20;28</sup> The results from this study also show that there was a wide inter-individual variation in the excretion of both 19-NA and 19-NE in the urine.<sup>20;51;79</sup>

In conclusion, this study has confirmed that individuals free of nandrolone can produce small amounts of 19-NA and 19-NE in the urine. Furthermore, when all the data are combined and analyzed, musculoskeletal injury does not significantly affect the concentration of 19-NA and 19-NE in the urine of male subjects. However, when data are analysed individually, there may be a small chance that an athlete may exceed the IOC cut-off concentration for 19-NA in the urine after sustaining a musculoskeletal injury. The mechanism explaining this needs further investigation so that the IOC guidelines for a positive nandrolone test can be adjusted to reduce the risk of an athlete having a false positive test for nandrolone.

## CHAPTER 4

### SUMMARY AND CONCLUSION

In summary, in accordance with the aims of the thesis, the following can be concluded:

- To validate the International Olympic Committee's cut-off concentration for urine 19-NA of  $2 \text{ ng.ml}^{-1}$  in male subjects immediately after sustaining musculoskeletal injury.

It was further demonstrated that one of the male subjects, who was free of synthetic nandrolone, produced a 19-NA urine concentration of  $2800 \text{ pg.ml}^{-1}$  immediately after musculoskeletal injury. This 19-NA concentration exceeded the cut-off concentration of  $2000 \text{ pg.ml}^{-1}$  set by the IOC. This may represent a 1 in 32 chance that an athlete who has sustained musculoskeletal injury after competition may produce a false positive nandrolone drug test result.

Based on the results from this study it is recommended that further similar studies be conducted to investigate the possible relationship between severe physiological stress and the excretion of nandrolone metabolites in the urine. It is also recommended that consideration be given by the IOC to use more sophisticated analytical techniques to distinguish endogenous from synthetic nandrolone in drug testing laboratories. This study highlights that it may be unethical to drug test competitive athletes after sustaining severe musculoskeletal injury and that other testing methods could be used as adjuncts to confirm nandrolone administration, namely isotope ratio mass

spectrometry, hair analysis<sup>23</sup> or analysis for the particular type of the steroid conjugate.<sup>49</sup>

- To determine whether moderately active male subjects who sustain severe physiological stress (musculoskeletal injury) can excrete nandrolone metabolites (19-NA and 19-NE) in the urine in higher concentrations compared to baseline.

The concentration of 19-NA and 19-NE in the urine of moderately active male subjects did not change significantly in all subjects after musculoskeletal injury when compared to baseline.

- To confirm the endogenous production of 19-NA and 19-NE in the urine of moderately active male subjects.

This study confirmed the endogenous production of small amounts of 19-NA and 19-NE in the urine of male populations who have not administered nandrolone.

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## APPENDICES

- Appendix A:** Informed consent – English.
- Appendix B:** Informed consent – Xhosa.
- Appendix C:** Informed consent – Afrikaans.
- Appendix D:** Exclusion Criteria.
- Appendix E:** Demographic information and health assessment.
- Appendix F:** Training history and training diary.
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- Appendix H:** Ethics approval letter from the University of Cape Town.

## Appendix A



### Informed consent form

*MRC/UCT Research Unit for Exercise Science and Sports Medicine,  
Department of Human Biology,  
University of Cape Town,  
Sports Science Institute of South Africa*

I \_\_\_\_\_ am fully aware that my injury will be diagnosed and treated according to standard medical practice at Milnerton Medi-Clinic. I am also aware that I will need to:

- Submit a urine sample for hormone testing (nandrolone metabolites).
- Submit a blood sample for hormone testing (cortisol, luteinizing hormone).

The possible risks and complications of a blood test have been explained to me. I am aware that I may withdraw my consent and stop taking part in the research study at any time.

I understand that the information collected may be used for scientific purposes and publication but that my confidentiality will be respected at all times during the study and when the report is published.

I understand the implications of my informed consent and any questions I may have had have been answered to my satisfaction.

Name: \_\_\_\_\_ Signed: \_\_\_\_\_ Date: \_\_\_\_\_

Researcher: \_\_\_\_\_ Signed: \_\_\_\_\_ Date: \_\_\_\_\_

Witness: \_\_\_\_\_ Signed: \_\_\_\_\_ Date: \_\_\_\_\_

## Appendix B:



### Ifomu yesivumelwano esazisayo

MRC/UCT Iyuniti Yezophando KwinzuluwaziyoThambo Nobugqirha bezemidlalo  
Isebe le Nzululwazi ya Buntu  
IYunivesithi yaseKapa  
Iziko le Nzululwazi laseMzantsi Afrika

Mna \_\_\_\_\_ ndaziswe ngokupheleleyo ngohlobo lolu phando kwaye ndinika imvume yokuba ndisetyenziswe kolu phando.

Ndiqonda ngokupheleleyo iinkqubo ezisetyenziswayo:

- Ukunikezela ngomchamo ukuvavanya incindiyedlala (nandrolone metabolites).
- Ukunikezela ngegazi ukuvavanya yedlala (cortisol, luteinizing hormone).

Ndicaciselwe ngobungozi nobumzima bokuthatha uvavanyo lwegazi. Ndiyaqonda ukuba ndingarhoxa kwisivumelwano kwaye ndiyeke ukuthatha inxaxheba kolu phando naninina.

Ndiyaqonda ukuba ulwazi oluqokelelweyo lungasetyenziswa kwinjongo zenzuluwazi nokushicilelwa ngendlela eyimfihlo.

Ndiqonda nesibophelelo sesi sivumelelwano esazisayo kwaye imibuzo ebendinayo iphendulwe ngendlela endonelisayo.

Igama \_\_\_\_\_ Sayina \_\_\_\_\_ Umhla \_\_\_\_\_

Umphandi \_\_\_\_\_ Sayina \_\_\_\_\_ Umhla \_\_\_\_\_

Ingqina \_\_\_\_\_ Sayina \_\_\_\_\_ Umhla \_\_\_\_\_

*\*This document has been translated by Mrs Rose Smouse, Lecturer, Department of African Languages, University of Cape Town.*

Appendix C:Toestemmingsform

Ek \_\_\_\_\_ is ten volle ingelig oor die beginsels van hierdie studie en gee hiermee my vrywillige toestemming om as proefpersoon aan die studie deel te neem.

Ek is ten volle bewus van die betrokke prosedures wat van my verwag word naamlik:

- Verskaffing van 'n urienmonster vir hormoontoetsing( nandrolone metabolites).
- Verskaffing van 'n bloedmonster vir hormoontoetsing (cortisol, luteinizing hormone).

Die potensiele risiko's en komplikasies van die bloedtrekprosedure is aan my verduidelik. Ek is bewus daarvan dat ek ten enige van hierdie studie kan onttrek.

Ek verstaan dat die ingesamelde informasie vir wetenskaplike doeleindes gebruik sal word en in 'n konfidensiële manier hanteer en gepubliseer sal word.

Ek verstaan die implikasies van my ondertekende toestemming en voel tevrede dat al my vrae ten volle beantwoord is.

Naam: \_\_\_\_\_ Geteken: \_\_\_\_\_ Datum: \_\_\_\_\_

Navorsers: \_\_\_\_\_ Geteken: \_\_\_\_\_ Datum: \_\_\_\_\_

Getuie: \_\_\_\_\_ Geteken: \_\_\_\_\_ Datum: \_\_\_\_\_

*\* This document was kindly translated by Miss Amanda Claassen.*

## Appendix D:



### Exclusion criteria

- Age younger than 18 years and older than 50 years.
- Female.
- Sedentary lifestyle.
- Injury severity score (ISS) > 13.
- Participation in competitive bodybuilding or power lifting.
- Previous use of prohormone supplements.
- Previous use of androgenic anabolic steroids.
- Previous positive drug test in sport and suspension from sports competition.
- Abnormal blood screening parameters (luteinizing hormone).
- Medical illness (asthma, cardiovascular disease, renal disease, prostate disease, breast cancer, liver disease).
- Medications known to affect steroid hormone concentrations e.g. all forms of hormone replacement therapy.

**Appendix E:****Demographic information and health assessment**

Subject number:
-----------------

Name: \_\_\_\_\_

Address: \_\_\_\_\_

\_\_\_\_\_ code \_\_\_\_\_

Phone: \_\_\_\_\_ (w) \_\_\_\_\_ (h) \_\_\_\_\_ (cell)

Email: \_\_\_\_\_

Age (years): \_\_\_\_\_

Height (cm): \_\_\_\_\_

Body mass (kg): \_\_\_\_\_

**Anthropometric data:**

- Thigh girth (cm) \_\_\_\_\_
- Thigh skinfold (mm) \_\_\_\_\_
- Calf girth (cm) \_\_\_\_\_
- Calf skinfold (mm) \_\_\_\_\_
- Forearm girth (cm) \_\_\_\_\_
- Forearm skinfold (mm) \_\_\_\_\_

**Medical history**

How did the injury occur (mechanism)? \_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

Where is the injury (body part)? \_\_\_\_\_

Describe the symptoms in full: \_\_\_\_\_

\_\_\_\_\_

What time did the injury occur? \_\_\_\_\_

What time were the blood and urine samples taken? \_\_\_\_\_

Were you exercising at the time?  Yes  No

If  Yes, please give details (type of activity, duration, intensity): \_\_\_\_\_

\_\_\_\_\_

Do you have any allergies?  yes  no

If yes, please explain: \_\_\_\_\_

\_\_\_\_\_

Are you using any medications?  yes  no

If yes, please explain: \_\_\_\_\_

\_\_\_\_\_

Do you smoke?  yes  no

If yes, state the number/day: \_\_\_\_\_

Describe any previous surgical procedures:

\_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

Do you have any medical illnesses? \_\_\_\_\_



## Appendix F:



### Training history and training diary

Training history: (last 3 months)

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Endurance training:

Average duration per day (min) in the last 3 months: \_\_\_\_\_

Average number of times per week in the last 3 months: \_\_\_\_\_

Resistance training:

Average duration per day (min) in the last 3 months: \_\_\_\_\_

Average number of times per week in the last 3 months: \_\_\_\_\_

Training diary:

Day	Date	Duration	Intensity	Type
Monday				
Tuesday				
Wednesday				
Thursday				
Friday				
Saturday				
Sunday				

## REVIEW

## Urine nandrolone metabolites: false positive doping test?

R M N Kohler, M I Lambert

*Br J Sports Med* 2002;**36**:325-329

The aim of this review is to analyse the studies on nandrolone metabolism to determine if it is possible for an athlete to test positive for nandrolone without having ingested or injected nandrolone.

The anabolic androgenic steroid 19-nortestosterone, also called nandrolone, was first synthesised by Birch in 1950.<sup>1</sup> Nandrolone has an anabolic effect, and is used in the treatment of certain chronic diseases.<sup>1,2</sup> The use of nandrolone by athletes became popular in the late 1950s.<sup>4</sup> Athletes use nandrolone in an oral or injectable form to increase muscle strength and improve performance.<sup>5</sup> As a result of the potential performance enhancing benefits<sup>4</sup> and potential health risks associated with anabolic steroid use,<sup>7</sup> the International Olympic Committee (IOC) prohibited the use of nandrolone in sport in 1976.

"A doping offence for nandrolone was defined as a concentration of NA in human urine exceeding 2 ng/ml in men and 5 ng/ml in women"

When nandrolone is ingested or injected by humans subjects, three metabolites of nandrolone can be isolated and measured in the urine by gas chromatography-mass spectrometry. These metabolites have been identified as 19-norandrosterone (NA; 3 $\alpha$ -hydroxy-5 $\alpha$ -oestrane-17-one), 19-noretiocholanolone (3 $\alpha$ -hydroxy-5 $\beta$ -oestrane-17-one), and 19-norepiandrosterone (3 $\beta$ -hydroxy-5 $\alpha$ -oestrane-17-one).<sup>8-13</sup> These metabolites are isomeric compounds, having the same chemical composition and molecular mass but different chemical structure. NA is usually the most abundant urine metabolite of nandrolone.<sup>8-13</sup> The presence of these metabolites in the urine forms the basis of doping analysis for the illegal use of nandrolone by athletes.<sup>8,12,14-16</sup> This was based on the premise that these urine metabolites could only have been derived from exogenous nandrolone. A study in 1982 appeared to have found NA, or a similar compound, in the urine of athletes who had not used nandrolone.<sup>8</sup> In 1996, the IOC stated that a critical concentration for nandrolone metabolites in the urine had been established. A doping offence for nandrolone was defined as a concentration of NA in human urine exceeding 2 ng/ml in men and 5 ng/ml in women.<sup>17</sup>

"Recently, the possibility of false positive tests for nandrolone has been raised."

Recently, the possibility of false positive tests for nandrolone has been raised. Explanations for false positive tests have included supplement contamination<sup>18</sup> and endogenous production of nandrolone and regulation of metabolic pathways of nandrolone metabolism by various physiological factors and supplement interventions. The aim of this review is to analyse the studies on nandrolone metabolism with the overall goal of determining whether it is indeed possible for an athlete to test positive for nandrolone without having either ingested or injected nandrolone. The question of a positive test resulting from nutritional supplements<sup>14,16</sup> and food contamination<sup>19,20</sup> is beyond the scope of this review.

## EVIDENCE FOR ENDOGENOUS NA

The origin of endogenous NA in the urine of athletes who have not knowingly ingested or injected nandrolone is central to resolving the question of whether it is possible to have a false positive test. The first study to suggest that NA could be found in the urine of people free of exogenous nandrolone was a study on laboratory staff ( $n = 14$ ).<sup>8</sup> Their urine was analysed using isotope dilution mass spectrometry, and NA or a similar compound was suspected. This suspicion was based on the detection of a small peak for the ion at  $m/z$  256.<sup>8</sup> In retrospect, this signal may have been caused by interference of other endogenous compounds (noise) and perhaps represents a false positive finding. The authors acknowledged the limitations of the study; the analytical technique lacking specificity and sensitivity.

Studies in 1988 and 1990 again raised the possibility of endogenous NA in the urine of humans. Kicman and Brooks<sup>21</sup> used radioimmunoassay and measured NA in the urine of men and women, who were supposedly free of exogenous nandrolone, ranging from 3.8 to 49.4 ng/ml. However, these data should be interpreted with caution as it could be argued that the analytical technique again lacks both specificity and sensitivity. Debruyckere *et al*<sup>11</sup> measured NA in the urine of three subjects at concentrations of 9, 14, and 37 ng/ml.<sup>11</sup> These results were later attributed to nandrolone contaminated meat which the subjects may have eaten.<sup>19,22</sup>

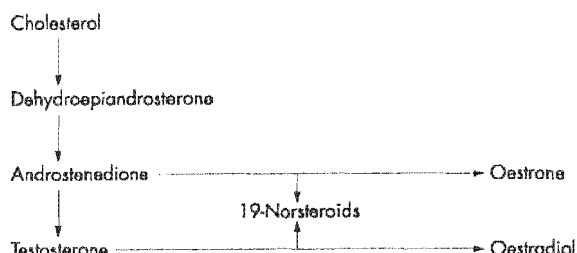
In 1996, the IOC declared that the presence of a small amount of NA in the urine was not considered to constitute a doping offence.<sup>17</sup> This suggests that they acknowledged the possibility

**Abbreviations:** IOC, International Olympic Committee; NA, 19-norandrosterone; GC-CIRMS, gas chromatography-combustion-isotope ratio mass spectrometry

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Accepted 26 February 2002



**Figure 1** The androgen biosynthetic pathway and the proposed aromatisation of androgen to oestrogen with the formation of 19-norsteroid intermediates.

of endogenous NA production. It can only be assumed that this decision was reached on the basis of data collected by IOC laboratories during routine drug testing, as the scientific evidence at the time was equivocal. In the late 1990s, analytical procedures for the detection and quantification of steroid metabolites in urine had become increasingly sensitive.<sup>22</sup> This may have accounted for an appreciable number of positive urine samples for NA being analysed in certain anti-doping laboratories. Many of the positive samples were from participants of sports that had previously not been associated with anabolic steroid use.<sup>1</sup> Further research with more sensitive equipment was undertaken to determine if NA could be produced naturally by the human body. This research showed convincing evidence that NA was found in the urine of subjects free of exogenous nandrolone.<sup>1, 15, 16, 24-28</sup> The urine NA concentrations in these studies ranged from 0.01 to 1.79 ng/ml. In a study by Galan Martin *et al.*,<sup>29</sup> high NA concentrations in five sportspeople (4, 5, 6, 8, and 14 ng/ml) were measured. One woman in the study, who was postmenopausal, had a NA concentration of 22 ng/ml. It could be argued that these athletes had administered nandrolone.<sup>29</sup> These results are difficult to explain and perhaps further investigation of these subjects is necessary before a definite opinion can be formed.

## METABOLISM OF NA

### Aromatisation

Metabolic pathways for the endogenous production of NA in the human body need to be considered. Under normal circumstances, testosterone is aromatised to oestrogen by the aromatase enzyme complex.<sup>30</sup> Androstenedione, the direct precursor of testosterone, is also aromatised to oestrogen by the aromatase enzyme.<sup>31, 32</sup> The important step in this metabolic process is the removal of the methyl group from the 19th carbon of either testosterone or androstenedione. Nandrolone differs structurally from testosterone and androstenedione in lacking the methyl group at the 19th carbon, and it is additionally different from androstenedione in substitution of a ketone group for an hydroxy group at the 17th carbon. Is it feasible that 19-norsteroids (nandrolone and metabolites) are intermediates in the aromatisation process? (fig 1).

Animal studies, *in vitro* experiments, and observations in humans, particularly pregnant women, add support to the proposal that 19-norsteroids are intermediates in the aromatisation of androgens to oestrogen.<sup>6, 15, 16, 21, 25, 26, 33-37</sup> Oestrogen concentrations in women increase significantly both at the time of ovulation and during pregnancy.<sup>26, 29</sup> Raised urine NA concentrations have recently been identified in women at the time of ovulation<sup>26, 29</sup> and during pregnancy.<sup>28</sup> Mareck-Engelke *et al.*<sup>29</sup> reported that during pregnancy the concentration of NA in human urine may reach 20 ng/ml. In these cases, pregnancy is confirmed with a blood test for human chorionic gonadotrophin.

A recent study by Reznik *et al.*<sup>35</sup> examined the sequelae of giving human chorionic gonadotrophin to 10 men. Human chorionic gonadotrophin increases serum testosterone in healthy men<sup>41-43</sup> and stimulates the aromatase enzyme, causing a gradual increase in serum oestrogen.<sup>44, 45</sup> The serum testosterone and oestrogen increased in the 10 subjects after human chorionic gonadotrophin administration, and NA excretion in the urine increased by 250%. It may be concluded from this study that the increase in nandrolone biosynthesis was possibly associated with the increased aromatisation of testosterone to oestrogen.

"Factors that could increase the flux of androgen precursors through the testosterone biosynthetic pathway could theoretically increase the amount of nandrolone produced."

Although the pathways proposed are theoretical, the available evidence suggests that it is possible for the flux of androgen precursors through the testosterone biosynthetic pathway to result in the production of endogenous nandrolone. Therefore it can be assumed that factors that could increase the flux of androgen precursors through the testosterone biosynthetic pathway could theoretically increase the amount of nandrolone produced.

## FACTORS WITH THE POTENTIAL TO AFFECT NA METABOLISM

### Genetics

There is a wide range of serum testosterone concentrations in men,<sup>32</sup> suggesting large genetic interindividual and intraindividual variability in sex steroid production and excretion over a 24 hour period.<sup>46</sup> The possibility therefore exists that there is a variable rate of NA excretion.<sup>1</sup> Indeed, endogenous NA urine excretion in a male athlete varied by 680% over a three month period<sup>1</sup> and in another subject by 72% over a 24 hour period.<sup>15</sup> The enzyme complex 17 $\beta$ -hydroxysteroid dehydrogenase, which is responsible for converting androstenedione into testosterone, and the aromatase enzyme complex, which converts testosterone into oestrogen, occur in muscle and fat.<sup>47</sup> Therefore, it is conceivable that people with higher muscle and fat content may be more proficient in the production of 19-norsteroid intermediates. The aromatase enzyme complex per se can also show considerable genetic variability in expression and activity in certain people,<sup>48</sup> with increased activity of the aromatase enzyme producing larger amounts of oestrogen. This prompts the question of whether genetic upregulation of the aromatisation process in these people increases the production of 19-norsteroids.

### Exercise

Intense exercise has been associated with raised levels of NA in the urine.<sup>1, 17, 26</sup> Le Bizec *et al.*<sup>26</sup> studied professional soccer players over 19 months and collected 385 urine samples. Urine NA concentrations after soccer games were significantly higher than before games. For NA concentrations after games, 70% of the urine samples were below 0.1 ng/ml, and 20% were between 0.1 and 0.2 ng/ml. NA in four urine samples were above 1.0 ng/ml, the maximum value being 1.79 ng/ml.<sup>26</sup> When urine is tested for banned substances and the specific gravity of the urine sample is measured above 1.020, urine metabolite concentrations are adjusted by a correction factor.<sup>33</sup> This analysis is based on the premise that urine flow rate and urine metabolite excretion remain constant during and directly after exercise. However, this is an erroneous assumption as it has been shown that excretion of pseudoephedrine after exercise was increased in subjects in whom urine volume remained constant.<sup>49</sup> Thus urine metabolite excretion may not remain constant during and directly after exercise, and

random urine sample collection after exercise may be unreliable.<sup>15, 42</sup> A more accurate measure would be to collect a urine sample over a 24 hour period, allowing the calculation of excretion rates of urine metabolites.<sup>15</sup> However, this is not practical, particularly when testing for drug use in sport.

The serum androgen response to exercise in athletes can vary according to the type, duration, and intensity of the exercise task.<sup>33-36</sup> Serum concentrations of testosterone, androstenedione, and dehydroepiandrosterone increase with short term, intense exercise as the result of increased testicular production by an unknown mechanism.<sup>37</sup> An increase in serum testosterone after exercise may also be caused by a decrease in the plasma volume<sup>38</sup> or a decrease in hepatic clearance.<sup>34</sup> The effect of exercise on serum oestrogen is also extremely variable.<sup>33</sup>

*"A urine specimen collected after high intensity exercise could have a higher concentration of NA"*

It is conceivable that the increase in circulating androgens in people participating in short duration, high intensity exercise could result in the stimulation of the aromatase enzyme complex, resulting in an absolute increase in the amount of NA in the urine. Therefore, there are sufficient data<sup>17, 26</sup> to suggest that a urine specimen collected after high intensity exercise could have a higher concentration of NA for reasons other than dehydration.

#### Trauma and hypoglycaemic stress

As yet, no study has investigated the possible effect that traumatic stress (musculoskeletal injury) may have on 19-norsteroid metabolism. Interestingly, two international male athletes, one an international rugby player<sup>39</sup> and the other a paraOlympian (B Frasure, personal communication), recently tested positive for NA above 2 ng/ml, after both having suffered significant injuries just before passing a urine sample for drug testing. Both athletes claimed to be innocent of a doping offence. The concentration of NA in the urine samples of both athletes was about 6 ng/ml, which is slightly above the IOC cut off concentration for men (2 ng/ml).

Reznik *et al.*<sup>23</sup> have provided some insight into the effect of a stress response on nandrolone metabolism. Hypoglycaemia was induced in 10 subjects by intravenous injection of 0.1 IU/kg insulin. Urine samples were collected at 0-2, 2-4, and 4-10 hours after the insulin injection. They concluded that hypoglycaemic stress did not significantly alter NA excretion. However, inspection of their data shows that, in certain subjects, NA excretion increased in the first two hours after induction of the hypoglycaemic stress. Had the study included more than 10 subjects, it is likely that there would have been sufficient statistical power to show that the increase in NA in the first two hours after hypoglycaemic stress would have produced a significant finding. Hypoglycaemic stress is associated with the production of glucose counter-regulatory hormones: cortisol, glucagon, growth hormone, and adrenaline.<sup>33</sup> Cortisol is produced in the adrenal cortex when stimulated by adrenocorticotrophic hormone. The latter also stimulates the production of androgens and mineralocorticosteroids from the adrenal cortex.<sup>32</sup> It is tempting to speculate that the increased production of adrenal androgens results in increased NA excretion as described above. Further studies need to evaluate whether the increase in adrenal androgens and their aromatisation could produce any changes in NA excretion after traumatic musculoskeletal stress.

#### Mineral cofactors and herbal products

There is also a theoretical argument that certain substances not prohibited in sport may alter nandrolone metabolism. For example, the trace element zinc is a cofactor in many enzymic processes in the body. An increase in serum testosterone in

men who are marginally zinc deficient has been shown after zinc supplementation.<sup>40</sup> Also, diets deficient in zinc resulted in a significant decrease in serum testosterone concentration. Therefore it can be concluded that zinc supports testosterone production.<sup>40</sup> Although there is a linear relationship between serum zinc and serum testosterone concentrations,<sup>41</sup> it is not known whether supraphysiological doses of zinc are associated with higher levels of testosterone production. Certain athletes are marginally zinc deficient<sup>42</sup> because of inadequate intake<sup>43</sup> and considerable sweat loss.<sup>44, 45</sup> As zinc status may not be optimal in these athletes, can zinc supplementation enhance testosterone production and could this increase in testosterone production increase the production of aromatisation intermediates? This question was partially addressed when a zinc/magnesium supplement (30 mg zinc) was given to football players nightly for eight weeks. This treatment increased free and bound serum testosterone by about 33%.<sup>46</sup> These findings were not attributed to haemoconcentration because the blood samples were taken 24 hours after exercise. On the basis of the possibility that 19-norsteroid metabolism may be associated with testosterone metabolism and the aromatisation process, it is feasible that zinc supplementation, combined with exercise, may increase nandrolone metabolites in the urine.

The herbal product tribulus terrestris (tribestan), which has been used in Eastern cultures since ancient times to treat impotence and improve libido, is another substance that has been associated with an increased serum testosterone concentration (S Milanov, unpublished work). Could tribestan in combination with exercise increase NA in the urine? Further research is necessary.

#### CHALLENGING THE IOC CUT OFF CONCENTRATION FOR URINE NA

Until recently, studies involving large numbers of subjects to determine the physiological range for the concentration of NA in the urine of men and women free of exogenous nandrolone were lacking. Data on the range of NA could only be drawn from the analysis of urine from sedentary and recreational people at rest.<sup>1, 15, 16, 24, 25</sup> The total number of subjects from all these studies is about 150. No specific mention is made in any of the studies of the age of the male subjects. This is relevant because testosterone production decreases with advancing age.<sup>47</sup> Therefore one might expect 19-norsteroid production to decrease also with advancing age, making the age of study populations an important consideration. The amount of NA in the urine from the subjects did not exceed 1 ng/ml, except in the study of Galan Martin *et al.*,<sup>23</sup> the concerns in which have already been raised.

*"There is no explanation by the IOC of why the threshold concentration for NA is higher in women."*

Two recent studies involving larger numbers of sportsmen have provided further evidence. Urine samples collected after exercise in these studies showed that the concentration of NA in the urine increased, and in certain men the concentration of NA was close to the cut off concentration of 2 ng/ml.<sup>27, 28</sup> Should this be combined with other stressors and possible supplement interventions (mentioned above), the concentration of NA in the urine is most unpredictable.

The IOC have also apparently collected data and measured NA urine excretion in elite male and female athletes at the 1996 Nagano Olympic games, but these data have not been released into the public domain.<sup>28</sup> It would be beneficial for the IOC data to be made public to support reasoning behind the calculation of cut off concentrations for NA in the urine of men and women. There is also no explanation by the IOC of why the threshold concentration for NA is higher in women. If

the reason for it is the higher circulating levels of oestrogen, particularly at the time of ovulation, is this not indirect support for the presence of 19-norsteroids as intermediates in the aromatisation of androgens to oestrogen?<sup>68</sup> Bradford-Hill has stated: "It is the essence of science to disclose both the data upon which a conclusion is based and the methods by which the conclusion is obtained".<sup>69</sup>

The IOC has defended the status quo on nandrolone and confirmed these threshold values of 2 ng/ml in men and 5 ng/ml in women in Monaco in October 1999.<sup>70</sup> The conditions of strict liability are currently applied in the case of any athlete contravening the above thresholds.

#### METHODS TO TEST FOR NA

A solution to the controversy surrounding nandrolone in sport is to develop a testing procedure that can accurately differentiate endogenous nandrolone metabolites from nandrolone that is ingested or injected. The technique of gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) to calculate the <sup>13</sup>C/<sup>12</sup>C ratio is currently being developed as a method to fulfil this purpose.<sup>1, 11-13</sup> This is based on the principle that natural steroids have a different carbon isotopic signature from synthetic steroids. The <sup>13</sup>C/<sup>12</sup>C ratio for synthetic nandrolone metabolites is lower than that for endogenous metabolites, therefore administering exogenous nandrolone will lower this ratio. This ratio has also been proposed as a method of detecting the use of synthetic testosterone as an alternative to the testosterone/epitestosterone ratio.<sup>11-13</sup> However, a potential problem with GC-C-IRMS is the lack of reproducibility and sensitivity because of the low levels of endogenous nandrolone metabolites present in the body. At present, this method can only be applied to "high" concentrations of NA (60 ng/ml) in the urine.<sup>13</sup>

Le Bizac *et al*<sup>14</sup> has proposed examining the steroid conjugates as an additional criterion to distinguish between the endogenous or exogenous origin of nandrolone metabolites. Endogenous NA was found to be 30% sulpho-conjugated in contrast with administered nandrolone, which was found to be 100% conjugated to glucuronic acid when excreted in the urine.<sup>14</sup>

Kintz *et al*<sup>15</sup> proposed that analysis of hair samples from athletes is another option to consider for detecting the presence of exogenous nandrolone. The analysis of hair samples could be used to accurately verify positive results obtained by gas chromatography-mass spectrometry.

Until the hair sample and GC-C-IRMS techniques have been validated on a large scale, a prudent approach after the detection of NA in urine samples above the cut off concentration is for the athlete to have further blood tests before the sample is declared positive, as is done for athletes with a high testosterone/epitestosterone ratio.

#### CONCLUSION

The abuse of the steroid testosterone presented a new problem for drug control in sport.<sup>12</sup> Perhaps the same can now be said for nandrolone. According to the Olympic movement anti-doping code, NA is not a prohibited substance.<sup>71</sup> However, should NA in the urine exceed a certain threshold concentration, the interpretation is that nandrolone has been ingested or injected. There is strong scientific evidence to show that NA can appear in the urine of people free of exogenous nandrolone. Evidence suggests that NA may occur as an intermediate in the aromatisation of testosterone to oestrogen. Recent evidence has shown that the amount of NA in the urine can be regulated by the administration of human chorionic gonadotrophin. Therefore, threshold concentrations for men (2 ng/ml) and women (5 ng/ml) as defined by the IOC are still open to debate because conclusive scientific evidence showing how these values may be altered by various physiological stimuli is lacking. In accordance with this,

#### Take home message

19-Norandrosterone (NA) is produced endogenously as an intermediate in the aromatisation of androgen to oestrogen. Intense exercise may increase its concentration in the urine. Future laboratory testing methods need to distinguish endogenous from exogenous nandrolone metabolites accurately, as this has important implications for doping control in sport.

multicentre studies need to answer further specific questions on the current urine threshold concentrations for nandrolone metabolites and whether physiological stressors and permitted supplement interventions can alter NA excretion.

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8 March 2002

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Prof MI Lambert  
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Dear Prof Lambert

**CAN ACUTE SEVERE MUSCULOSKELETAL TRAUMA AFFECT THE EXCRETION OF  
NANDROLONE METABOLITES IN THE URINE**

Thank you, for submitting your study to the Research Ethics Committee for review.

It is a pleasure to inform you that the Committee has **formally approved** your study.

**Please quote above Rec reference number in all correspondence**

Yours sincerely

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