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Excretion of 19-norandrosterone after consumption of boar meat

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Abstract

The consumption of the offal of noncastrated pigs can lead to the excretion of 19-norandrosterone (NorA) in urine of humans. In doping control, GC/C/IRMS is the method of choice to differentiate between an endogenous or exogenous origin of urinary NorA. In some cases, after the consumption of wild boar offal, the $\delta^{13}\text{C}$ values of urinary NorA fulfill the criteria of an adverse analytical finding due to differing food sources of boar and consumer. However, consumption of wild boar's offal is not very common in Germany, and thus, the occurrence of such an analytical finding is unlikely. In contrast, the commerce with wild boar meat has increased in Germany within the last years. Up to 20,000 tons of wild boar meat are annually consumed. In order to probe for the probability of the occurrence of urinary NorA after consumption of wild boar meat, human urine samples were tested following the ingestion of commercially available game. In approximately half of the urine samples, traces of NorA were detected postadministration of 200 to 400 g boar meat. The highest urinary concentration was 2.9 ng/ml, and significant amounts were detected up to 9 h after the meal. $\delta^{13}\text{C}$ values ranged from -18.5‰ to -23.5‰ , which would have led to at least two adverse analytical findings if the samples were collected in an antidoping context. IRMS analysis on German boar tissue samples showed that $\delta^{13}\text{C}$ values for wild boar's steroids are unpredictable and may vary seasonally.

KEYWORDS

boar meat, carbon isotope ratios, norandrosterone

1 | INTRODUCTION

In doping control analysis, isotope ratio mass spectrometry (IRMS) is used to distinguish between an endogenous or exogenous origin of the urinary 19-norsteroid norandrosterone (NorA).^{1,2} In humans, NorA can be naturally found in urine in small amounts (<2 ng/ml) formed, for example, by demethylation of androsterone (A) and, at higher concentrations, during pregnancy.^{2–4} NorA can also be formed in urine by in situ microbial degradation of A.^{4,5} In addition, NorA is the main urinary metabolite of the therapeutic nortestosterone (NT, nandrolone)

as well as of prohormones such as 4-norandrostenediol, 5-norandrostenediol, or 4-norandrostenedione (NAED), which are all prohibited in sports.^{5,6} A source of so-called “pseudo-endogenous” urinary NorA can be the consumption of the offal of noncastrated pigs.^{5–8}

In boars, the highest concentrations of 19-norsteroids can be found in testicles (NAED up to 84 $\mu\text{g}/\text{kg}$, NT up to 172 $\mu\text{g}/\text{kg}$), liver, and kidney.⁹ Much lower but nevertheless significant amounts have been found in boar meat (NAED up to 0.9 $\mu\text{g}/\text{kg}$, NT up to 3.6 $\mu\text{g}/\text{kg}$).⁹ Although the consumption of boar offal is not particularly common in Germany, the consumption of wild boar meat is increasing

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within the last years with around 600,000 wild boars harvested per hunting season,¹⁰ corresponding to approximately 10,000 to 20,000 tons of wild boar meat being consumed in Germany every year.¹¹

We have reported previously that the carbon isotopic composition of free-ranging ("wild") boar tissue and steroids in Germany may vary seasonally and can result in $\delta^{13}\text{C}$ values from -13‰ to -24‰ .⁸ The forage of free ranging boars is usually dominated by C_3 -plants (wheat, barley, acorns, etc.) in winter and spring, but as soon as maize crops are available, the boars rely elusively on this C_4 -plant as primary forage. Thus, the consumption of offal but also potentially meat can lead to the urinary excretion of NorA with a carbon isotopic signature different from the consumer. This "pseudo-endogenous" origin of NorA may lead to adverse analytical findings in doping control testing if $\delta^{13}\text{C}$ values of consumer and consumed animal differ more than 3‰.⁵

It has been stated improbable that concerning antidoping tests, significant amounts of urinary NorA in humans may originate from the consumption of boar meat,¹² and if so, the corresponding $\delta^{13}\text{C}$ values of such urinary NorA would be "endogenous-like."⁴ As these assumptions are yet to be corroborated, an excretion study was conducted to verify or falsify the possibility of an adverse analytical finding after consumption of boar meat.

2 | EXPERIMENTAL

2.1 | Test meals and participants

The meals were prepared using varying amounts of boar meat products (Table 1), with weights ranging from 187 to 491 g (prepared weight). The products were randomly selected and obtained from butcheries or online distributors. Each volunteer consumed one meal, with time and side dishes being arbitrary. Nine male volunteers with

an average body weight of 90.4 ± 8.9 kg and three female volunteers (67.0 ± 5.6 kg) were included in the study. The participants were requested to collect one urine sample prior to the meal and all urine samples for a period of 24 h after meat consumption. Samples were stored at $+4^\circ\text{C}$ until analysis. The participants gave written informed consent prior to the study. Test meals were not checked for NorA content or carbon isotope composition.

2.2 | Sample preparation of urine for GC/MS/MS

The samples were prepared according to the laboratory internal standard operating procedure for anabolic steroids.¹³ Conjugated and unconjugated steroids were extracted from urine at pH 9.6 with *tert*-butylmethyl ether (TBME, in-house purified by distillation) following enzymatic hydrolysis of the glucuronides at pH 7 (β -glucuronidase, Roche). After centrifugation, the organic layer was transferred and evaporated to dryness. The dry residue was derivatized with 100 μl of *N*-methyl-*N*-trimethylsilyl trifluoroacetamide (MSTFA, Macherey-Nagel)/ammonium iodide (Sigma-Aldrich)/ethanethiol (Merck, for synthesis) (v:v:v, 1000:2:3).

2.3 | GC/MS/MS

The GC/EI-MS/MS experiments were performed in accordance to earlier protocols¹⁴ using a Trace 1310 gas chromatograph interfaced to a TSQ 8000 triple quadrupole mass spectrometer (all Thermo Scientific). The GC system was equipped with an Ultra1 capillary column (length 17 m, i.d. 0.2 mm, film thickness 0.1 μm , Agilent) in split (1:10) mode. The initial GC oven temperature was 184°C , increasing at $3^\circ\text{C}/\text{min}$ to 232°C and at $40^\circ\text{C}/\text{min}$ to a final temperature of 310°C .

TABLE 1 Type and amount of test meals including maximum urinary NorA concentrations and $\delta^{13}\text{C}$ values after consumption for all participants (m = male, f = female)

	Type	Amount/g	[NorA] _{max} /ng/ml	NorA/NorE	$\delta^{13}\text{C}_{\text{ERC}}/\text{‰}$	$\delta^{13}\text{C}_{\text{NorA}}/\text{‰}$	$ \Delta\delta /\text{‰}$
1 (m)	Roast ^a	187	<1	3.5	-22.9	-19.7	3.2
2 (m)	Roast ^b	491					
3 (m)	Roast ^a	410	2.9	7.6	-24.3	-18.5	5.8
4 (m)	Roast ^a	200					
5 (f)	Roast ^a	200					
6 (m)	Ham ^a	300	2.5	4.9	-23.3	-23.5	0.2
7 (f)	Canned meat ^a	220	<1	4.0	-22.8	-21.4	1.4
8 (f)	Canned meat ^a	215	<1	4.3	-23.8	-21.9	1.9
9 (m)	Canned meat ^c	235					
10 (m)	Canned meat ^c	240					
11 (m)	Jerky ^c	90/(315) ^d					
12 (m)	Jerky ^c	120/(420) ^d					

^aObtained from a meat market, Northern Germany.

^bLocal supplier, Southern Germany.

^cOnline distributor, Estonian origin.

^dNumber in brackets represents the corresponding raw weight of the dried meat.

Helium (4.6, Linde) was used as carrier gas (0.9 ml/min, constant pressure) and argon (5.0, Linde) as collision gas. The injector and interface temperatures were both set to 300°C, and the ion source was operated at 250°C. Ionization was accomplished using electron ionization (EI) (70 eV).

2.4 | Sample preparation of urine for GC/C/IRMS

Preparation of the urine samples (20–30 ml) followed the laboratory internal standard operating procedure¹⁵ that comprised the following steps: solid-phase extraction (Chromabond C18, 500 mg, 6 ml, Macherey-Nagel) with methanol (LC grade, J.T. Baker) followed by a liquid–liquid extraction with TBME (GC grade, Merck), an enzymatic hydrolysis with β -glucuronidase (Roche) at pH 7, and a second liquid–liquid extraction with n-pentane (pa, Merck) at pH 9.6. By means of two different HPLC runs, the steroids of interest were separated and fractionated. The first HPLC was a reversed-phase purification on a XBridge RP18 5 μ m column, followed by a second HPLC employing a XBridge C18 column 5 μ m (both columns from Waters). The fractions were dried and acetylated for IRMS.

2.5 | GC/C/IRMS of urinary NorA

Samples were measured per GC/C/IRMS on a Trace 1310 gas chromatograph equipped with an HP-5MS chromatographic column and coupled via a ConFlo IV to a MAT 253 isotope ratio mass spectrometer (all Thermo Scientific). Carbon isotope ratios are expressed in per mill relative to VPDB. Monitoring gas (CO₂, purity 4.5, Linde) was scale calibrated using acetylated steroid mixtures USADA 33-1 (Cornell University, Ithaca, NY) comprising 5 α -androstan-3 β -ol acetate, 5 α -androstan-3 α -ol-17-one acetate, 5 β -androstan-3 α -ol-11,17-dione acetate, and 5 α -cholestane¹⁶ and CU/PCC 44-1 (Cornell University, Ithaca, NY), which comprises 5 α -androstan-3 α -ol-17-one acetate, 5 β -androstan-3 α ,17 β -diacetate, 5 α -cholestane, and 5 β -pregnan-3 α -20 α -diacetate. Accuracy of the instrument was checked using quality control charts for all steroids analyzed in every sequence, as well as using the internal working standard 5 α -androstan-3 β -ol acetate. Sample preparation was checked using negative and positive control samples according to the WADA technical document.⁵

2.6 | EA/IRMS of bristles

Bristles were washed using distilled water and soaked for 30 min in methanol (Roth)/chloroform (pa, Merck; v:v, 2:1) in an ultrasonic bath (Bandolin Sonorex). After drying, they were cut into 10 mm segments. As a multi-isotopic analysis (CHNS) was performed, and due to the limited sample volume, only two to three segments per animal were analyzed for $\delta^{13}\text{C}$. Between 1.8 and 2.0 mg of bristles were weighed into tin capsules in quadruplicate. Samples were

analyzed at isolab GmbH, Schweitenkirchen, Germany, using a Vario EL Cube elemental analyzer (Elementar Analysensysteme) connected to a mass spectrometer (Isoprime). Internal standards used during analysis were casein (Kremer Pigmente) and two different horse tail hair samples (both from local suppliers). Scale calibrations were performed with NBS 22 (–30.03‰, IAEA) and IRMM-BCR 657 (–10.76‰, IRMM). The analytical precisions using at least quadruplicate measurements were $\delta^{13}\text{C} = \pm 0.1\text{‰}$. The data were drift corrected using repeated measurement of laboratory standard after 30 samples.

3 | RESULTS AND DISCUSSION

3.1 | Urinary concentrations of NorA

No NorA was detected in the “blank” urine samples sampled prior to ingestion of the boar meat. Therefore, endogenous production of NorA of the volunteers can be excluded. NorA was detected in urine samples of five of the 12 volunteers (Table 1). Two of them exhibited significant amounts of NorA with maximum concentrations of 2.9 and 2.5 ng/ml (1.9 and 2.1 ng/ml after adjustment for specific gravity),⁵ whereas in the urine samples of the other three volunteers, only traces of NorA lower than 1 ng/ml were found (Figure 1). No NorA was detected in the urine samples of the remaining seven volunteers. Peak NorA concentrations were reached after 3.3 to 6.0 h after consumption of the boar meat. All urine samples from volunteers that showed traces of urinary NorA tested negative 24 h after the meal.

For all urine samples, also the concentration of nortiocholanolone (NorE) was determined. The NorA/NorE ratio, which is an indicator of an exogenous administration of 19-norsteroids,⁵ was increased (>3) in all urine samples with NorA concentrations greater than 0.5 ng/ml. The results for NorA concentrations and NorA/NorE ratios of this study were similar to those found in another studies after ingestion of boar's offal.^{7,17}

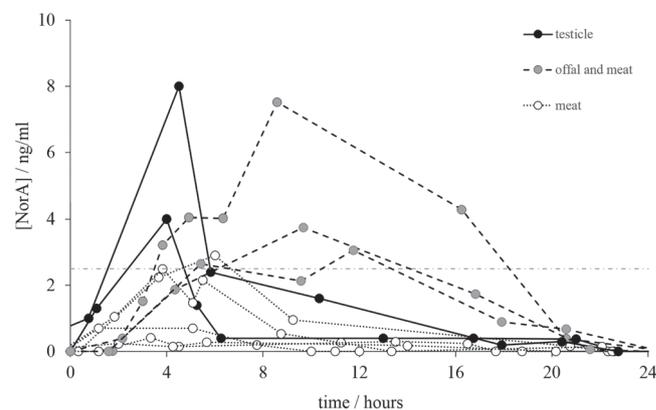


FIGURE 1 Excretion profiles of urinary NorA after consumption of boar testicles (white circles),⁷ mixed meals of boar meat and offal (grey circles),¹⁷ and boar meat (black circles). The horizontal line reflects the 2.5 ng/ml cut-off level for mandatory IRMS analyses

3.2 | $\delta^{13}\text{C}$ of urinary NorA

For each volunteer those urine samples with the detectable NorA concentrations were further analyzed by GC/C/IRMS. Pregnanediol (PD) was analyzed as endogenous reference compound (ERC) with $\delta^{13}\text{C}$ values around -24.3‰ and -22.8‰ and, thus, in good agreement with typical values observed for German inhabitants.¹⁸ Urinary NorA showed $\delta^{13}\text{C}$ values between -23.5‰ and -18.5‰ (Table 1). Accordingly, the absolute differences between ERC and NorA as defined in the relevant technical Document of WADA ranged from $|\Delta\delta| = 0.2\text{‰}$ to 5.8‰ for the volunteers. Hence, urine samples of two study participants yielded $|\Delta\delta| > 3$, which would be considered as adverse analytical findings (AAF) according to currently enforced regulations.⁵

All urine samples with traces of NorA resulted from the ingestion of boar meat obtained from the same meat market in Northern Germany; however, not all meals prepared from meat originating from that market ($n = 7$) led to an excretion of urinary NorA. It is assumed that the meat was derived from different animals, as the products were either purchased at different times and/or the best-before dates

were not identical. Further, deviating $\delta^{13}\text{C}$ values of the urinary NorA indicate different sources of the metabolite's precursor.

3.3 | $\delta^{13}\text{C}$ of boar's bristles

Bristles of seven wild boars hunted in Germany were analyzed per EA/IRMS. Two wild boars originated from Northern Germany, one boar was from in North-Rhine Westphalia (data have already been published before⁸), and four boars were from southern Germany (Figure 2). The wide range of $\delta^{13}\text{C}$ values within the bristles of one individual, published earlier,⁷ were confirmed by the herein conducted additional analyses, although the intraindividual variation differed between the animals. There was neither any spatial difference in $\delta^{13}\text{C}$ detectable nor any difference according to age.

Almost all boars showed a dietary shift from C_3 to C_4 -plant based diets (or vice versa) within their bristles. It is known that the $\delta^{13}\text{C}$ values of mammalian (and human) body protein, either hair keratin or muscle protein, rapidly adapts toward a change in the ^{13}C content of the diet.^{19–22} According to these studies, $\delta^{13}\text{C}$ values for mammalian

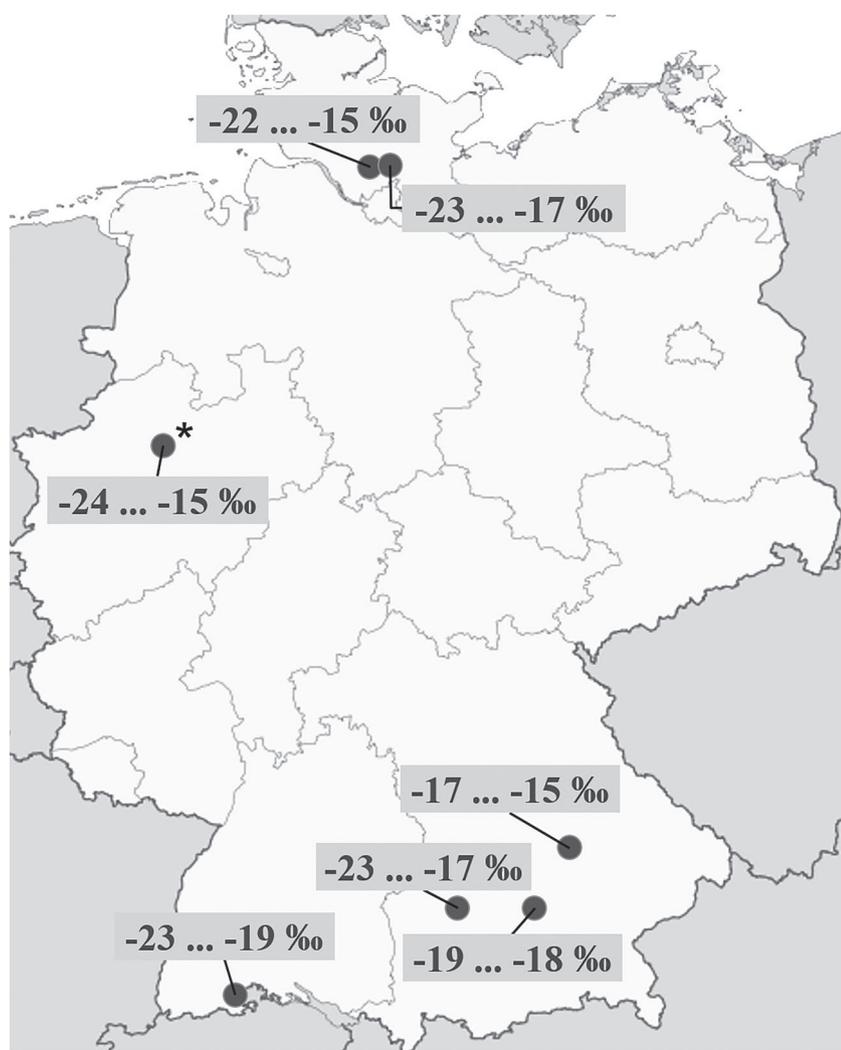


FIGURE 2 Range of $\delta^{13}\text{C}$ values of wild boar's bristles in Germany. Each circle with corresponding $\delta^{13}\text{C}$ values represents one individual at its origin. Values for individual marked with an asterisk (*) are from a previous publication⁸

hair of -15% can be attributed to an almost completely corn-based diet.^{19,20,22} On the other hand, $\delta^{13}\text{C}$ values of -24% and lower are attributed to exclusively C_3 -plants in the diet.^{19,20,22}

The wide range of $\delta^{13}\text{C}$ values of about 10% found in boar's bristles correspond to values found for urinary NorA after consumption of boar meat or offal, which range from -13% to -24% . From diet experiments, it is known that human protein (hair keratin) and endogenous steroids adapt concurrently to dietary changes (C_3 - to C_4 -plant based food or vice versa).^{21,23} The absolute $\delta^{13}\text{C}$ values of steroids and hair protein are comparable, although individual offsets up to 2.5% exist, with the urinary steroids being, in most of the cases, more depleted in ^{13}C than hair protein.

3.4 | Interpretation of human urinary NorA

Our results confirm the hypothesis that low urinary NorA concentrations in humans can be the result of the consumption of meat from uncastrated male pigs. Although the concentration of 19-norsteroids in wild boar meat is lower than in offal such as testicles or liver, the consumption of a typical meal of wild boar meat can lead to urinary concentrations around 2 ng/ml (after adjustment for specific gravity).⁵ In order to distinguish between an endogenous or exogenous origin according to TD2019NA for urinary concentrations of NorA greater than 2.5 ng/ml (after adjustment for specific gravity), IRMS is mandatory; below 2.5 ng/ml , IRMS is optional. Due to the varying and unpredictable diets of wild boars (in Germany) $\delta^{13}\text{C}$ values of urinary NorA can present values from -15% to -24% . As typical endogenous values for steroids of a German population are around -23% ,^{8,18,21,24} urinary NorA deriving from wild boar meat may be interpreted as "endogenous," if the boar's diet was C_3 -based; conversely, it can be identified as "exogenous" if the animal was on a C_4 -based diet. According to TD2019NA, an absolute difference between ERC and NorA greater than 3% has to be reported as an adverse analytical finding.⁵

To our knowledge, synthetic pharmaceutical preparations of 19-norsteroids exhibit $\delta^{13}\text{C}$ values between -33% and -21% .^{25,26} Thus, to date, urinary NorA presenting more enriched $\delta^{13}\text{C}$ values is more likely an indication for boar meat (or offal) ingestion than for the administration of a synthetic 19-norsteroid.

The fact of varying $\delta^{13}\text{C}$ values of wild boar could also be problematic for people living in countries with a high consumption of C_4 -plants like the United States or Southern Africa. Human endogenous $\delta^{13}\text{C}$ values in these countries are enriched in ^{13}C compared with Germany, and for these, there is the possibility of adverse analytical findings after the consumption of the meat of 19-norsteroid producing C_3 -fed boars.

Moreover, it appears unlikely that after the consumption of boar meat urinary concentrations of NorA rise above 15 ng/ml , which is the upper cut-off level for a GC/C/IRMS target analysis of suspicious samples.⁵ The highest urinary concentration of NorA after consumption of boar meat in our study was 2.9 ng/ml (410 g of prepared

meat). Higher urinary concentrations of NorA may be found after consumption of offal, or mixed meals of meat and offal.^{7,8,17}

4 | CONCLUSION

The herein presented results show that detectable amounts of NorA may occur in human urine after ingestion of wild boar meat. As it has been shown previously, it is unpredictable which $\delta^{13}\text{C}$ values of urinary NorA can be expected after such a meal. It is still advisable to avoid the term "endogenous" for $\delta^{13}\text{C}$ values of 19-norsteroid metabolites of a free ranging animal in comparison with $\delta^{13}\text{C}$ values of human urinary steroids. It has been confirmed that the $\delta^{13}\text{C}$ values for animal 19-norsteroids vary between -13% and -25% and do not necessarily correlate with $\delta^{13}\text{C}$ values found for human individuals of the same geographical region. Not only the consumption of wild boar's offal in the hours preceding a doping control test but also the consumption of wild boar meat may result in an atypical or even positive test result, albeit the urinary NorA concentrations are expected to be lower than after consumption of wild boar's offal. Both athletes as well as anti-doping laboratories and authorities should still be aware of this aspect.

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