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Title page

uPA$^{+/+}$-SCID mouse with humanized liver as a model for *in vivo* metabolism of 4-androstene-3,17-dione.

Leen Lootens, Peter Van Eenoo, Philip Meuleman, Geert Leroux-Roels, and Frans T. Delbeke

Doping Control Laboratory (DoCoLab), Ghent University, Department of Clinical Chemistry, Microbiology and Immunology, Zwijnaarde, Belgium (L.L., P.V.E., F.T.D.); Center for Vaccinology, Ghent University and Hospital, Department of Clinical Chemistry, Microbiology and Immunology, Ghent, Belgium (P.M., G.L.R.)

Running title page

a) 4-androstene-3,17-dione metabolism in chimeric mice

b) Corresponding author
Leen Lootens
DoCoLab, Ghent University,
Technologiepark 30 B
9052 Zwijnaarde
Belgium
Tel: +32 (0)9/3313293
Fax: +32 (0)9/3313299
E-mail: Leen.Lootens@UGent.be

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d) Nonstandard abbreviations
AD= 4-androstene-3,17-dione
DHEA= dehydroepiandrosterone
DHT= Dihydrotestosterone
GC-MS= gas chromatography-mass spectrometry
hAlb= Human Albumin
LOD= Limit of detection
LOQ= Limit of quantification
PBS= phosphate buffered saline
RSD= relative standard deviation
uPA-SCID= urokinase Plasminogen Activator-Severe Combined Immuno Deficiency

ABSTRACT

The metabolism in primary human hepatocyte cultures often deviates from clinical studies, which in turn are hampered by ethical constraints. Here the use of uPA$^{+/+}$-SCID mice transplanted with human hepatocytes was investigated as a model for *in vivo* metabolic studies. The urinary excretion profile after oral administration of 4-androstene-3,17-dione (AD) in chimeric mice was investigated using gas chromatography-mass spectrometry detection and was compared to previously reported metabolites of AD in humans and cell cultures. Chimeric mice exhibited a similar AD metabolic profile as humans, showing androsterone and etiocholanolone as major metabolites. Several hydroxylated steroids were detected in the chimeric mice as minor metabolites, when compared with hepatocyte cultures. A significant correlation between the extent of liver replacement and the relative abundances of human-type metabolites was established. The results for AD showed that humanized liver-uPA-SCID mice can serve as an alternative model for *in vivo* metabolism studies in humans. In the future this model could possibly be used for other steroids or pharmaceutical compounds.

INTRODUCTION

The liver is one of the most important organs involved in the metabolism of drugs (Katoh et al., 2008). Most orally administered steroids are metabolized in the liver and excreted in the urine. The misuse of anabolic androgenic steroids in sports is controlled by evaluating the presence of prohibited substances or their metabolites in urine samples. Since experimental *in vivo* human excretion studies are not always approved by an ethical committee, the metabolism of steroids is mainly studied in primary human hepatocyte cultures. However, these *in vitro* experiments do not always accurately predict the *in vivo* metabolic profile (Schänzer, 1996; Lévesque and Ayotte, 1999; Uralets and Gillette, 1999; Goudreault et al., 2001; Leder et al., 2001; van de Kerkhof, 2001; Lévesque et al., 2002; Brandon et al., 2003; Van Eenoo and Delbeke, 2006).

In this study a chimeric mouse model with functional human hepatocytes was evaluated as an alternative model for metabolic studies (Gonzalez 2003; Gonzalez and Yu, 2006). Urokinase-type plasminogen activator (uPA)-SCID mice suffer from a severe transgene-induced liver disease. When transplanted with primary human hepatocytes, up to 90 % of the diseased liver parenchyma can be replaced with functional hepatocytes of human origin (Tateno et al., 2004; Meuleman et al., 2005). The human albumin (hAlb) concentration, measured in the mouse plasma, is a good marker for the replacement index of the chimeric liver.

The expression of human drug metabolizing enzymes in these chimeric mice has already been investigated (Tateno et al., 2004; Katoh et al., 2004, 2005 a-b, 2007; Okumura et al., 2007; Emoto et al., 2008; Muruganandan and Sinal, 2008). Many induction and inhibition reactions typical for human cytochrome P450 enzymes in the liver have been successfully tested in the chimeric mouse. Cytochrome P450 enzymes are predominantly expressed in the liver and

play a central role in drug metabolism. Although these experiments demonstrated the activity of human drug metabolizing enzymes, the complete metabolic excretion profile has not been evaluated yet. The present results indicate the potential of the chimeric mouse as a model for *in vivo* metabolic studies of designer steroids, for which approval of an ethical committee for administration studies in humans is virtually impossible to obtain.

In the study described herein a validation of the human-type metabolic profile in chimeric mice with 4-androstene-3,17-dione (AD) as model steroid was performed. The complete metabolic profile of AD in chimeric mice after oral AD administration has been investigated. The gas chromatography-mass spectrometry (GC-MS) results are compared with data generated from hepatocyte cell cultures and human administration studies (Schänzer, 1996; Lévesque and Ayotte, 1999; Uralets and Gillette, 1999; Goudreault et al., 2001; Leder et al., 2001; van de Kerkhof, 2001; Lévesque et al., 2002; Brandon et al., 2003; Van Eenoo and Delbeke, 2006).

The endogenous anabolic androgenic steroid 4-androstene-3,17-dione (AD) is a precursor of testosterone (Leder et al., 2001; Lévesque et al., 2002; Van Eenoo and Delbeke, 2006) and is prohibited by the World Anti-Doping Agency (WADA, 2009). The use of steroids is prohibited in sports because of their performance enhancing effects. However, most anabolic steroids are produced for therapeutic use. Athletes, aware of the benefits of steroids on weight gain and muscle strength, take them in much higher doses than therapeutically advised. Generally, a daily dose of 100-300 mg for endogenous steroids is recommended by manufacturers, but higher doses are sometimes used by athletes (Leder et al., 2001; Bashin et al., 1996).

In humans, AD is mainly metabolised to androsterone and etiocholanolone (Figure 1). Androsterone and etiocholanolone can be considered as final products in the androgen degradation pathway, whereas testosterone and dihydrotestosterone (DHT) represent intermediates. The data obtained in this administration study show that the metabolic profile of AD in chimeric mice closely resembles that in humans.

METHODS

Animals. The project was approved by the Animal Ethics Committee of the Faculty of Medicine of the Ghent University (Ghent, Belgium; approval ECD 06/09). uPA-SCID mice were transplanted with primary human hepatocytes (chimeric mice), as described before (Meuleman et al., 2003). Chimeric mice (n=6) and non-chimeric mice (n=4) without transplanted human hepatocytes were used. The non-chimeric mice served as a control group. Prior to the administration experiments, the human albumin (hAlb) concentration was measured in the mouse plasma. Chimeric mice with different hAlb concentrations were selected in the study (Table 1).

Sample collection of the mouse urine. Special metabolic cages for small rodents were purchased from Tecniplast (Someren, The Netherlands). The mice had free access to powdered food and water during the experiments. Because of the design of the metabolic cages, the urine and the faeces of the mice were perfectly separated. The urine was collected daily, non-invasively and was stored at -20°C awaiting analysis.

Study design. In a pilot study, the detection time in post-administration urine and possible saturation of the metabolic pathways were tested in the mice via oral administration of increasing doses of AD (175, 350 and 600 µg).

The chimeric and non-chimeric mice were randomly assigned to one of the following groups: administration with 100 µL of the placebo or with 100 µL of the AD-phosphate buffered saline (PBS) suspension. The AD-PBS suspension was made by weighing pure reference standard of AD (Sigma, St. Louis, MO, USA) and afterwards dissolving it in 5 % ethanol (Biosolve, Valkenswaard, The Netherlands) and further diluting it with PBS (Invitrogen,

Merelbeke, Belgium) to reach the final concentration. For the double blind administration study both the steroid suspension and placebo were administered with a washout period of 72h in between. The placebo was used as an extra control in this study to exclude any stress related endogenous steroid production.

The steroid suspension or placebo were administered on 3 consecutive days by oral gavage. Urine samples were collected after 24 h on the day prior to the first administration and on days 1, 2 and 3. The urinary excretion rates per day were measured.

Analysis. GC-MS has proven to be a reliable analytical tool for the identification of AD and its metabolites in mouse urine. The results of the quantitative GC-MS method development and validation with the instrumental settings for GC-MS have been described (Lootens et al., 2008). Briefly, mouse urine samples (100 µL) containing 17α-methyltestosterone as internal standard were hydrolyzed and afterwards extracted with 5 mL diethyl ether (Biosolve, Valkenswaard, The Netherlands). The evaporated organic phase is derivatized and analyzed by GC-MS.

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RESULTS

Pilot study: dose selection. After a single oral administration of increasing doses of AD (175, 350 and 600 µg) to chimeric mice, the parent compound and several metabolites were detected up to 24 hours. No differences in metabolic profile were detected between the 175 and 350 µg dose. When 600 µg was administered, the relative concentration of the parent drug AD increased, indicating possible saturation of the metabolic pathways. Therefore, the ideal administered dose was set at 350 µg/gavage. This dose is, per kg bodyweight, 5 times higher than the normal therapeutically dose used in humans. AD or its metabolites were no longer detected after 24 h, hence multiple dosing (Day 1-2-3) was possible without interference or accumulative effects. Moreover, a washout period of 72 h was included between the administration of the steroid suspension and placebo.

Blank and placebo. Prior to the administration of AD, urine was collected from both non-chimeric and chimeric uPA-SCID mice. In both these groups, the endogenous steroids were undetectable or in the range of the LOD (Lootens et al., 2008). In addition, none of the monitored AD metabolites could be detected in the urine of placebo treated chimeric and non-chimeric mice. These placebo results indicate that there is no interference of endogenously produced steroids with the detected steroids after AD administration and that the oral gavage did not induce any detectable stress related steroid production.

Administration results. The urinary metabolite concentrations on day 1-2-3 after multiple AD administration in 6 chimeric mice and 4 non-chimeric mice are shown in Tables 1 and 2. Phase I metabolic reactions and phase II glucuronidation of steroids were checked in the same

way as is done for routine doping control analysis (Lootens et al., 2008). After analysing the mouse urine samples, the average concentration (n=3) per steroid and the relative standard deviation (RSD %) were calculated (Tables 1-2).

To make the data comparable, the individual metabolite concentrations of Tables 1-2 are set relative to the sum of all detected steroids (Figures 2-3). After AD administration, the metabolic pattern in the non-chimeric control mice was significantly different from the chimeric mice. The results for all non-chimeric mice showed similar patterns (Figure 3).

Method validation and the low RSD for the intraday measurements indicate a good analytical reproducibility (Tables 1-2). Moreover, an excellent interday repeatability (D1-D2-D3) of the relative abundances of the monitored steroids was observed (Figures 2-3). Hence, the apparent high interday variation in absolute concentrations of the detected steroids could only be attributed to the uncertainty related to the administered dose. Steroids have a low solubility in polar agents like PBS resulting in the formation of a suspension. Nevertheless, gavage of a suspension was still considered as the best possible option to administer the steroids properly, because other possible administration routes (e.g. mixed with feed) could lead either to contamination problems or to a higher variation in administered dose.

A significant variability in the relative abundances of testosterone and epitestosterone after AD administration between the chimeric mice was noticed (Figure 2). A higher epitestosterone than testosterone concentration was found in two chimeric mice (Chim 4 and 6). Uncertainty about the origin of epitestosterone still exists in man (Catlin et al., 2002; Starka, 2003) although it is of great interest because it is the denomination in the T/E ratio,

which is used in sports to monitor misuse of endogenous steroids. Catlin et al. (2002) previously reported that androstenedione administration affects epitestosterone metabolism in men. In this study, AD administration increased epitestosterone excretion in both types of mice and hence confirms the previously reported results.

As commonly known (van de Kerkhof, 2001), conversion of AD, a Δ4 steroid, to a Δ5 steroid like dehydroepiandrosterone (DHEA), does not occur in humans (Figure 1). The results for the chimeric and non-chimeric mice are in agreement with these observations, since DHEA was not detected in the mice (Tables 1-2).

DISCUSSION

In humans, the major urinary markers of AD administration are androsterone and etiocholanolone. Testosterone, epitestosterone, DHT, 5β-androstane-3α,17β-diol (Diol 1), 5α -androstane-3α,17β-diol (Diol 2) and 5α -androstane-3β,17β -diol (Diol 3) are also described as metabolites (Schänzer, 1996; Lévesque and Ayotte, 1999; Uralets and Gillette, 1999; Goudreault et al., 2001; Leder et al., 2001; van de Kerkhof, 2001; Catlin et al., 2002; Starka, 2003; Van Eenoo and Delbeke, 2006). Although major metabolites *in vitro* (Lévesque et al., 2002), 6α-hydroxyandrostenedione and several other hydroxylated steroids are reported as minor metabolites *in vivo* (Schänzer, 1996; Lévesque and Ayotte, 1999; Goudreault et al., 2001; van de Kerkhof, 2001; Lévesque et al., 2002; Van Eenoo and Delbeke, 2006). Therefore the 16 metabolites monitored in this study were divided in non-hydroxylated and hydroxylated metabolites (Figure 1).

In the non-chimeric mice 9 out of the 16 screened steroids were detected (Table 2). Within the group of non-hydroxylated metabolites only testosterone, epitestosterone and AD were detected (Table 2-Figure 1). Neither androsterone nor etiocholanolone, the major metabolites reported in humans, nor DHEA, DHT, Diol 1, 2 and 3 were detected. On the other hand, most hydroxylated metabolites were found (Table 2-Figure 1).

In the chimeric mice, a significant difference could be observed between the results before and after AD administration (Table 1). AD is almost completely metabolised, indicating that the metabolic enzymes are sufficiently present and no saturation occurred. The parameters of the steroid profile that were altered the most were androsterone and etiocholanolone concentrations (Table 1). Androsterone and etiocholanolone seem to be specific markers for

AD use in humans and chimeric mice but not in control non-chimeric mice. Urinary testosterone, epitestosterone and DHT were also significantly higher in chimeric mice. Based on previous studies investigating AD metabolism in humans (Schänzer, 1996; Lévesque and Ayotte, 1999; Uralets and Gillette, 1999; Goudreault et al., 2001; Leder et al., 2001; van de Kerkhof, 2001; Van Eenoo and Delbeke, 2006), the same metabolic pathways could be found in the chimeric mice. Both *in vivo* in humans and in the chimeric mice, androsterone and etiocholanolone are the major metabolites. However, the diols where detected in very low concentrations or even below the LOQ (Table 1).

Figure 4 shows an overview of the relative excreted amounts of hydroxylated and non-hydroxylated steroids for all chimeric and non-chimeric mice. The 6 chimeric mice are ranked according to their hAlb concentration, showing the effect of the replacement with human hepatocytes on drug metabolism by evaluation of the urinary AD excretion profile (Table 1). Previously Tateno et al. (2004) showed a positive correlation between the extent of replacement of the liver with human hepatocytes and the hAlb concentration. Hence the increase of non-hydroxylated metabolites in the chimeric mice can be explained by the increased replacement with human hepatocytes. Indeed, a positive correlation (r=0.82) was found between the plasma hAlb concentration and the relative amount of non-hydroxylated steroids excreted (Figure 4). This study confirms that the major human metabolites increase in chimeric mice in a hAlb concentration-dependent manner and lead to a good correlation with human *in vivo* metabolism.

In the non-chimeric mice the hydroxylated metabolites were most abundant (Figure 4). The non-chimeric mice give similar results as the previously described *in vitro* data, since the same major metabolic pathway (hydroxylation) is described. While chimeric mice show a high degree of correlation with previously reported *in vivo* studies of AD. Therefore this

novel chimeric mouse model offers a promising approach and it is complementary to other models (including *in vitro* metabolism models).

However, some limitations are involved with the chimeric mouse model. It is technically very challenging to successfully obtain these transplanted mice. Moreover high quality cryopreserved human hepatocytes are required to obtain a good success rate of the transplantation procedure (Meuleman and Leroux-Roels, 2008). Also for other compounds undergoing significant metabolism outside the liver, like in the small intestine or lungs the value of the mouse model is less.

In conclusion, this study shows that chimeric mice with a humanized liver can be used for the *in vivo* study of the human urinary excretion profile of steroids. In contrast to non-chimeric control mice and primary hepatocyte cultures, chimeric mice metabolised orally administered AD in a similar way as humans do. However, further research with other known steroids is necessary. The chimeric mouse model circumvent the ethical problems associated with the investigation of steroid metabolism in humans, which implicate that the detection of designer steroids is still a challenge in doping analysis. Potentially in the future this mouse model can be applied to identify and characterize metabolites of designer steroids and nutritional supplements currently abused by athletes. In addition, this chimeric mouse model could be used to study the pharmacokinetics and metabolism of new therapeutic agents.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1: Androstenedione metabolism, with indication which metabolites were found *in vivo* in humans (1), chimeric mice (2), non-chimeric mice (3) or *in vitro* in primary hepatocyte cultures (4).

Legend: Dehydroepiandrosterone (DHEA), 4-androstene-3,17-dione (AD), epitestosterone (EpiT), testosterone (T), dihydrotestosterone (DHT), etiocholanolone (E), androsterone (A), 5α/β-androstane-3α/β,17β-diol (Diol 1, 2 and 3), 4-hydroxyandrostenedione (4OH-AD), 6α-hydroxyandrostenedione (6αOH-AD), 16α-hydroxyandrostenedione (16αOH-AD), 6β-hydroxyandrosterone (6βOH-A), 6β-hydroxyetiocholanolone (6βOH-E) and 16α-hydroxyandrosterone (16αOH-A).

Figure 2: Metabolites detected in the 24 h urine after multiple AD administration (day 1-2-3) to 6 chimeric mice. The mean measured concentrations (n=3) are set relatively to the sum of all steroids = 100 %. The chimeric mice (Chim 1-6) are ranked according to the plasma human albumin concentration (mg/mL). Legend: see Table 1.

Figure 3: Metabolites detected in the 24 h urine after multiple AD administration (day 1-2-3) to 4 non-chimeric mice. The mean measured concentrations (n=3) are set relatively to the sum of all steroids = 100 %. Legend: see Table 1.

Figure 4: Overview of the relative abundances of hydroxylated and non-hydroxylated metabolites after AD administration to (A) non-chimeric (n=4) and (B) chimeric mice (n=6). The chimeric mice are ranked according to the plasma human albumin (hAlb) concentration (mg/mL).

TABLES

Table 1: Absolute concentrations of the metabolites detected after multiple administration (D1-D2-D3) of AD in 6 chimeric mice (Chim1-6).

Table 1

NON-HYDROXYLATED METABOLITES[a]											
		Concentrations (ng/mL), (n=3) ± RSD (%)									
hAlb (mg/mL)		**T**	**EpiT**	**E**	**A**	**DHT**	**AD**	**Diol 1**	**Diol 2**	**Diol 3**	**DHEA**
Chim$_1$	D1	270.7 ±9.4	37.9 ±1.9	451.4 ±5.4	370.4 ±4.1	42.1 ±0.6	-	52.8 ±10.1	-	-	-
0.4	D2	432.0 ±2.3	45.7 ±5.6	590.5 ±0.9	480.3 ±1.7	50.4 ±3.9	-	54.5 ±1.0	-	-	-
	D3	26.8 ±5.5	21.7 ±1.5	376.3 ±3.9	139.5 ±12.6	13.6 ±17.7	-	10.0 ±13.2	-	-	-
Chim$_2$	D1	348.5 ±3.4	54.8 ±3.0	336.6 ±4.2	228.6 ±4.0	44.8 ±9.2	18.7 ±13.3	53.2 ±6.7	-	-	-
0.5	D2	411.8 ±11.3	65.5 ±4.4	341.1 ±5.4	215.7 ±8.8	64.5 ±4.6	22.8 ±1.4	50.2 ±9.5	-	-	-
	D3	254.6 ±6.3	42.3 ±5.2	215.8 ±5.6	106.1 ±9.5	28.9 ±3.9	18.1 ±3.7	25.2 ±4.3	-	-	-
Chim$_3$	D1	1183.8 ±3.9	112.5 ±8.1	1360.2 ±6.7	1534.6 ±1.6	168.9 ±8.0	321.0 ±0.5	202.9 ±8.1	-	-	-
1.1	D2	321.0 ±3.2	85.8 ±7.2	873.5 ±2.5	769.1 ±5.1	43.2 ±9.6	93.0 ±3.9	97.0 ±4.2	-	-	-
	D3	395.2 ±1.5	130.1 ±7.1	1291.4 ±2.0	885.8 ±1.4	66.4 ±6.0	77.2 ±7.0	122.0 ±5.2	-	-	-
Chim$_4$	D1	193.4 ±3.9	1739.8±13.7	8368.8 ±9.7	1115.7 ±15.5	-	16.0 ±4.0	168.4 ±12.3	66.1±5.6	-	-
2.0	D2	34.7 ±5.1	712.7±6.4	2701.7 ±12.9	237.3 ±4.7	-	4.4 ±88.6	62.3 ±14.2	69.3 ±8.8	-	-
	D3	51.3 ±0.5	1007.3±17.8	3156.7 ±2.3	342.8 ±6.8	-	11.8 ±14.3	76.8 ±6.3	92.8 ±3.6	-	-
Chim$_5$	D1	233.5 ±3.6	129.0 ±7.6	2509.4 ±4.8	3412.1 ±4.1	-	12.1 ±18.4	115.2 ±7.2	55.2 ±12.8	-	-
2.7	D2	119.4 ±0.6	112.2 ±4.3	3803.6 ±0.7	2669.3 ±1.4	-	13.8 ±23.8	206.5 ±4.0	54.5 ±1.2	-	-
	D3	276.9 ±1.6	122.8 ±2.1	2142.6 ±5.4	2641.6 ±5.3	-	-	98.5 ±4.5	91.9 ±8.3	-	-
Chim$_6$	D1	67.9 ±4.9	183.9 ±2.8	2800.8 ±3.1	4917.2 ±2.3	41.8 ±10.0	19.2 ±6.8	85.7 ±2.2	113.0 ±8.4	-	-
4.5	D2	69.5 ±0.7	123.9 ±10.7	1933.4 ±0.8	1890.9 ±2.1	132.1 ±10.4	48.2 ±1.9	56.4 ±9.2	36.2 ±6.7	-	-
	D3	69.3 ±2.5	172.1 ±3.2	2729.4 ±3.3	3814.3 ±2.8	38.7 ±10.8	43.9 ±1.5	78.9 ±2.0	71.1 ±2.6	-	-

[a] ***Non-Hydroxylated metabolites****: testosterone (T), epitestosterone (EpiT), etiocholanolone (E), androsterone (A), dihydrotestosterone (DHT), 4-androstene-3,17-dione (AD), 5β-androstane-3α,17β-diol (Diol 1), 5α-androstane-3α,17β-diol (Diol 2), 5α -androstane-3β,17β -diol (Diol 3) and dehydroepiandrosterone (DHEA).*

Table 1

HYDROXYLATED METABOLITES[b]							
		Concentrations (ng/mL), (n=3) ± RSD (%)					
hAlb (mg/mL)		**6αOH-AD**	**4OH-AD**	**16αOH-AD**	**6ßOH-A**	**6ßOH- E**	**16αOH-A**
Chim$_1$	D1	184.8 ±4.5	403.7 ±9.2	719.4 ±9.8	148.9 ±8.9	25.5 ±7.7	574.0 ±14.7
0.43	D2	289.0 ±4.3	652.3 ±2.5	1376.3 ±3.1	238.3 ±8.0	53.4 ±4.6	523.7 ±8.8
	D3	26.8 ±12.3	146.9 ±19.6	358.3 ±13.8	57.7 ±14.1	19.3 ±7.4	118.3 ±15.4
Chim$_2$	D1	149.0 ±0.5	333.4 ±1.8	206.3 ±5.1	101.3 ±14.6	33.5 ±7.4	240.0 ±12.6
0.50	D2	285.4 ±1.8	424.4 ±3.5	369.2 ±3.2	97.6 ±5.7	42.2 ±4.7	167.4 ±3.6
	D3	147.4 ±3.6	289.3 ±9.7	304.4 ±5.5	46.0 ±4.6	26.4 ±12.4	161.7 ±13.5
Chim$_3$	D1	432.5 ±3.8	1536.7 ±6.3	826.3 ±12.9	275.7 ±11.9	150.8 ±12.4	867.4 ±9.8
1.1	D2	170.6 ±4.3	560.7 ±5.9	142.4 ±5.2	172.7 ±11.2	43.0 ±13.7	199.3 ±8.1
	D3	239.3 ±3.0	680.4 ±7.2	188.9 ±12.4	228.1 ±8.1	67.5 ±10.1	224.5 ±7.8
Chim$_4$	D1	254.8 ±5.7	549.5 ±7.4	818.8 ±5.8	1086.6 ±5.1	529.3 ±2.6	1584.2 ±9.9
2.0	D2	129.8 ±1.3	258.7 ±4.1	379.4 ±5.2	1016.8 ±5.9	206.4 ±5.4	781.0 ±8.1
	D3	242.2 ±1.8	418.8 ±2.2	528.5 ±4.4	1567.6 ±4.7	242.0 ±6.3	1137.8 ±12.0
Chim$_5$	D1	82.9 ±8.5	47.1 ±12.7	372.6 ±7.2	1433.0 ±6.1	152.7 ±1.7	485.3 ±8.3
2.7	D2	62.8 ±5.8	28.7 ±11.9	205.4 ±6.1	1981.6 ±3.4	95.0 ±10.6	218.6 ±4.6
	D3	75.0 ±11.1	29.4 ±18.2	269.9 ±5.4	988.8 ±8.6	115.4 ±1.8	356.9 ±6.4
Chim$_6$	D1	106.2 ±0.5	154.8 ±2.8	144.0 ±10.0	693.7 ±14.2	76.6 ±6.8	625.8 ±15.8
4.5	D2	55.1 ±1.2	78.0 ±1.3	93.6 ±3.4	386.5 ±8.1	33.4 ±8.2	286.6 ±3.1
	D3	156.6 ±1.8	137.2 ±0.5	129.3 ±5.8	526.0 ±8.3	64.3 ±3.4	567.8 ±1.0

[b] ***Hydroxylated metabolites****: 6α-hydroxyandrostenedione (6αOH-AD), 4-hydroxyandrostenedione (4OH-AD), 16α-hydroxyandrostenedione (16αOH-AD), 6β-hydroxyandrosterone (6βOH-A), 6β-hydroxyetiocholanolone (6βOH-E) and 16α-hydroxyandrosterone (16αOH-A).*

Table 2: Absolute concentrations of the metabolites detected after multiple administration (D1-D2-D3) of AD in 4 non-chimeric mice (Non-Chim 1-4).

NON-HYDROXYLATED METABOLITES[a]											
		Concentrations (ng/mL), (n=3) ± RSD (%)									
		T	**EpiT**	**E**	**A**	**DHT**	**AD**	**Diol 1**	**Diol 2**	**Diol 3**	**DHEA**
Non-Chim$_1$	D1	70.5 ±0.7	43.0 ±2.0	-	-	-	-	-	-	-	-
	D2	96.1 ±2.1	22.7 ±6.6	-	-	-	-	-	-	-	-
	D3	245.1 ±6.5	21.7 ±1.7	-	-	-	-	-	-	-	-
Non-Chim$_2$	D1	36.8 ±3.9	6.8 ±18.9	-	-	-	-	-	-	-	-
	D2	104.0 ±8.1	16.0 ±10.4	-	-	-	-	-	-	-	-
	D3	49.7 ±8.4	10.8 ±13.0	-	-	-	-	-	-	-	-
Non-Chim$_3$	D1	10.6 ±8.2	8.9 ±7.6	-	-	-	21.0 ±5.3	-	-	-	-
	D2	20.5 ±3.4	10.4 ±2.3	-	-	-	24.9 ±1.4	-	-	-	-
	D3	46.3 ±4.1	8.7 ±2.2	-	-	-	20.7 ±3.7	-	-	-	-
Non-Chim$_4$	D1	27.8 ±4.4	7.6±2.5	-	-	-	17.1 ±2.8	-	-	-	-
	D2	31.0 ±5.9	12.3±4.9	-	-	-	26.2 ±0.8	-	-	-	-
	D3	82.7 ±3.8	13.6±0.4	-	-	-	30.3 ±2.6	-	-	-	-

[a] ***Non-Hydroxylated metabolites****: testosterone (T), epitestosterone (EpiT), etiocholanolone (E), androsterone (A), dihydrotestosterone (DHT), 4-androstene-3,17-dione (AD), 5β-androstane-3α,17β-diol (Diol 1), 5α-androstane-3α,17β-diol (Diol 2), 5α -androstane-3β,17β -diol (Diol 3) and dehydroepiandrosterone (DHEA).*

Table 2

HYDROXYLATED METABOLITES[b]							
		Concentrations (ng/mL), (n=3) ± RSD (%)					
		6αOH-AD	**4OH-AD**	**16αOH-AD**	**6ßOH-A**	**6ßOH- E**	**16αOH-A**
Non-Chim$_1$	D1	36.1 ±6.5	65.4 ±0.5	249.8 ±8.0	85.7 ±2.8	27.7 ±2.1	77.6 ±2.3
	D2	46.9 ±4.1	88.3 ±4.0	299.3 ±7.8	141.6 ±4.1	76.5 ±0.6	108.6 ±3.4
	D3	77.7 ±2.6	177.1 ±7.5	495.0 ±11.0	214.5 ±10.9	100.2 ±9.1	180.2 ±8.7
Non-Chim$_2$	D1	19.0 ±13.4	78.8 ±2.0	78.4 ±4.8	202.2 ±3.1	81.9 ±7.1	-
	D2	31.5 ±1.5	64.6 ±15.4	85.4 ±11.3	248.7 ±12.8	57.0 ±6.4	-
	D3	26.3 ±3.5	67.0 ±10.1	90.3 ±4.9	148.1 ±17.6	50.2 ±15.2	-
Non-Chim$_3$	D1	19.5 ±5.3	33.7 ±4.5	83.7 ±11.3	105.4 ±5.2	94.3 ±6.1	101.9 ±9.2
	D2	29.2 ±8.9	47.9 ±3.2	96.8 ±2.2	79.9 ±2.9	71.7 ±2.5	84.0 ±4.0
	D3	25.3 ±4.8	57.8 ±2.4	216.5 ±6.0	80.6 ±3.6	79.4 ±2.3	92.4 ±9.4
Non-Chim$_4$	D1	18.4 ±5.3	15.2 ±4.4	73.2 ±3.3	73.0 ±13.4	50.6 ±14.9	71.9 ±4.3
	D2	35.3 ±2.0	54.6 ±0.7	86.7 ±13.4	158.4 ±7.7	92.4 ±5.7	145.5 ±1.2
	D3	38.3 ±2.7	68.0 ±0.9	162.1 ±0.7	157.9 ±6.6	105.0 ±3.3	132.2 ±1.3

b ***Hydroxylated metabolites****: 6α-hydroxyandrostenedione (6αOH-AD), 4-hydroxyandrostenedione (4OH-AD), 16α-hydroxyandrostenedione (16αOH-AD), 6β-hydroxyandrosterone (6βOH-A), 6β-hydroxyetiocholanolone (6βOH-E) and 16α-hydroxyandrosterone (16αOH-A).*

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Figure 1

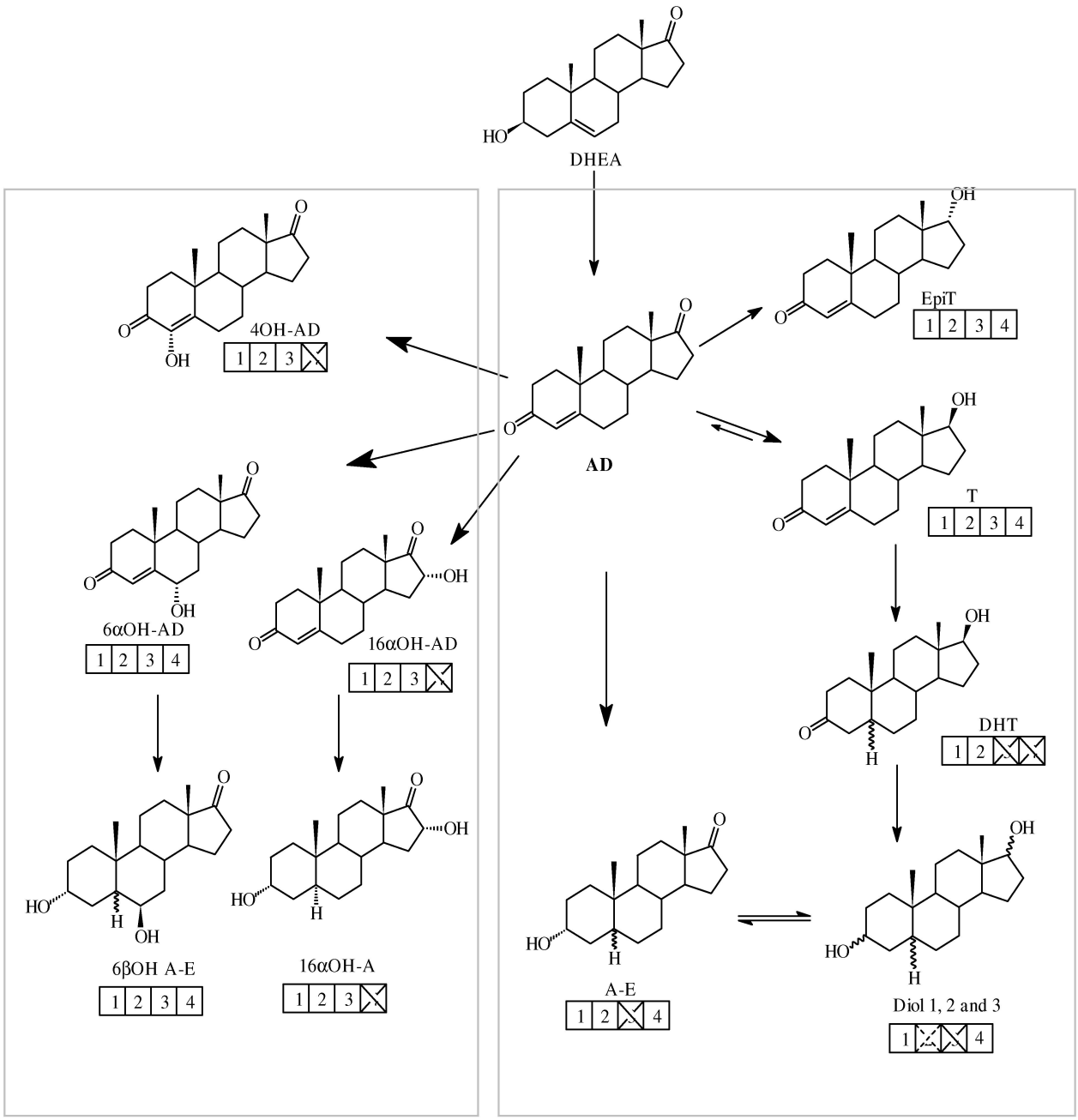

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Figure 2

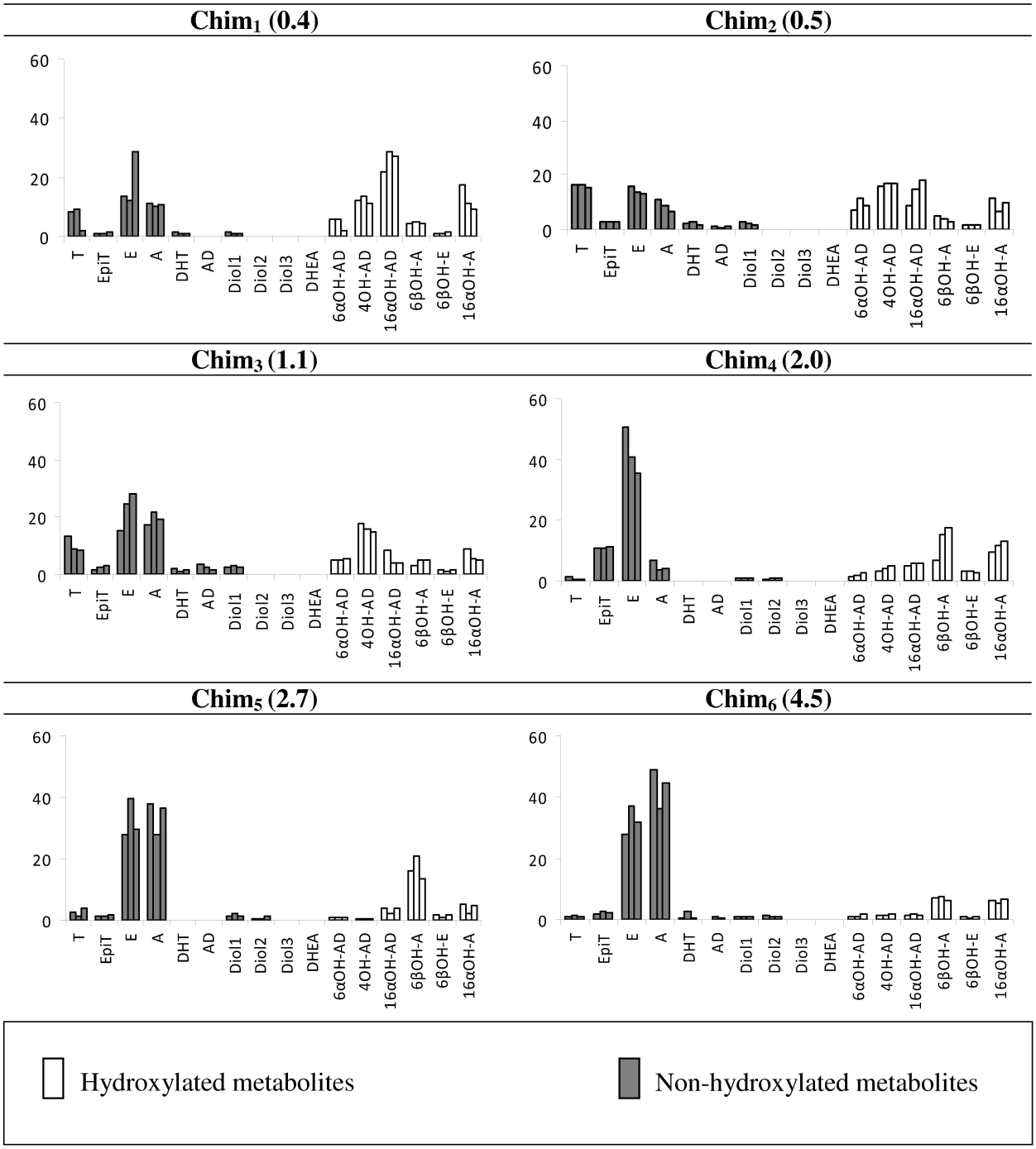

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Figure 3

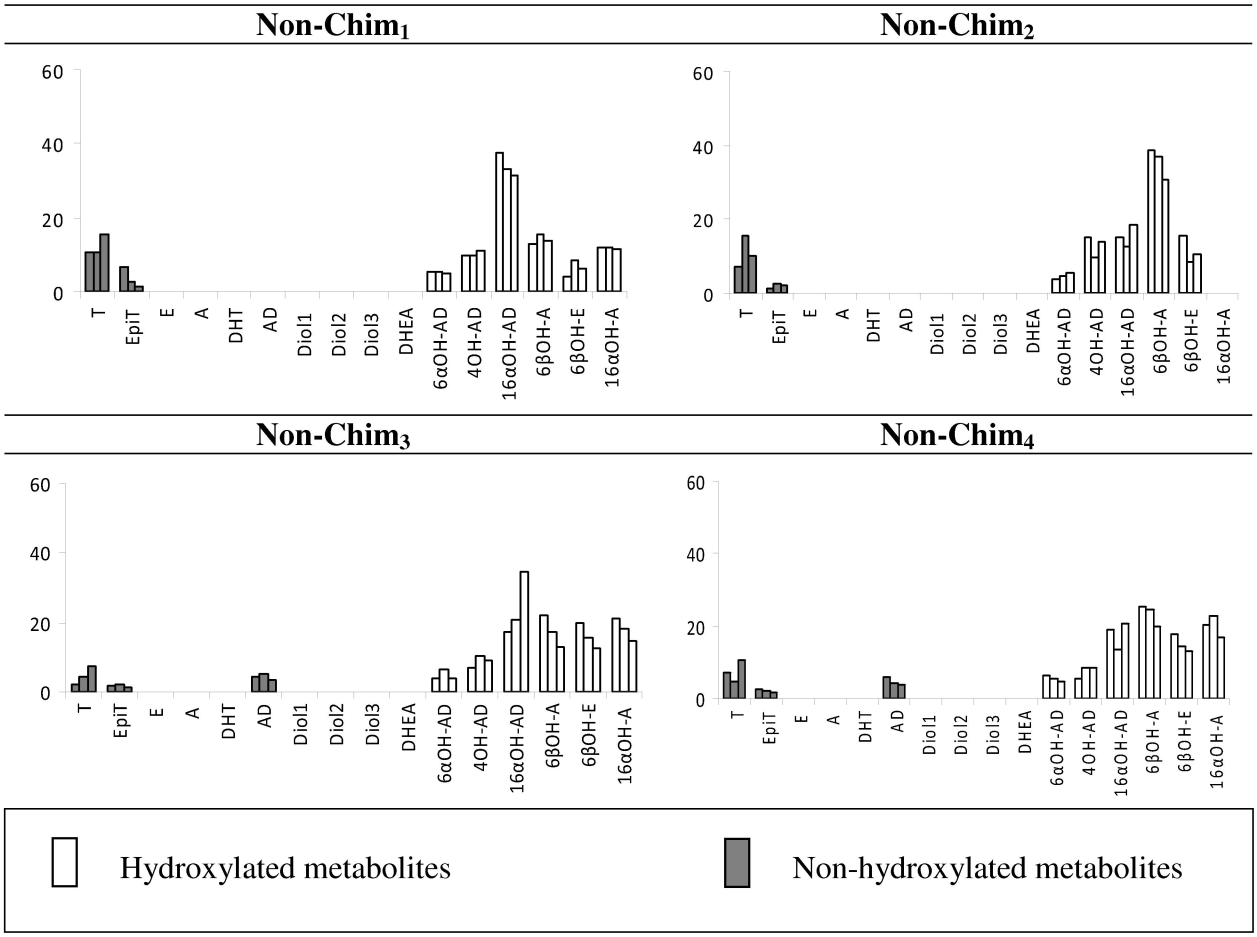

Figure 4