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Steroid Metabolomic Signature of Liver Disease in Nonsyndromic Childhood Obesity

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Abstract

Objective: Analysis of steroids by gas chromatography-mass spectrometry (GC-MS) defines a subject's steroidal fingerprint. Here, we compare the steroidal fingerprints of obese children with or without liver disease to identify the 'steroid metabolomic signature' of childhood non-alcoholic fatty liver disease.

Methods: Urinary samples of 85 children age 8.5-18.0 with BMI>97% were quantified for 31 steroid metabolites by GC-MS. The fingerprints of 21 children with liver disease (L1) as assessed by sonographic steatosis ($L1^{L}$), elevated alanine aminotransferases ($L1^{A)}$ or both ($L1^{AL}$), were compared to 64 children without markers of liver disease (L0). The steroidal signature of the liver disease was generated as the difference in profiles of L1 against L0 groups.

Results:L1 comparing to L0 presented higher fasting triglycerides (p=0.004), insulin (p=0.002), INS/GLU (p=0.003), HOMA-IR (p=0.002), GGTP (p=0.006), AST/SGOT (p=0.002), postprandial glucose (p=0.001) and insulin (p=0.011). $L1^{AL}$ showed highest level of T-cholesterol and triglycerides (p=0.029; p=0.044). Fasting insulin, postprandial glucose, INS/GLU and HOMA-IR were highest in $L1^{L}$ and $L1^{AL}$ (p=0.001; p=0.017; p=0.001; p=0.001). The liver disease steroidal signature was marked by lower DHEA and its metabolites, higher glucocorticoids (mostly tetrahydrocortisone) and lower mineralocorticoid metabolites than L0. L1 patients showed higher 5α-reductase and 21-hydroxylase activity (the highest in $L1^{A}$&$L1^{AL}$) and lower activity of 11βHSD1 than L0 (p=0.041, p=0.009, p=0.019). **Conclusions**: The 'steroid metabolomic signature' of liver disease in childhood obesity provides a new approach to the diagnosis and further understanding of its metabolic consequences. It reflects the derangements of steroid metabolism in NAFLD that includes enhanced glucocorticoids and deranged androgens and mineralocorticoids.

Introduction

Nonsyndromic childhood obesity is associated with nonalcoholic fatty liver disease (NAFLD), a spectrum of conditions, ranging from steatosis to nonalcoholic steatohepatitis (NASH), and various degrees of fibrosis and cirrhosis (1). NAFLD is regarded as the hepatic manifestation of the metabolic syndrome (2). However, childhood obesity with no NAFLD is also complicated by the metabolic syndrome. Despite the growth of knowledge regarding obesity-related NAFLD in children, we still rely mostly on circulating levels of liver enzymes and ultrasonography imaging and some non-invasive tests (3,4,5,6,7). Liver biopsy in children with suspected NAFLD is recommended only for "those where the diagnosis is unclear, where there is possibility of multiple diagnoses, or before starting therapy with potentially hepatotoxic medications" (8).

The consequences of obesity-related NAFLD on liver metabolism are insufficiently understood (8). As steroid hormones are partially catabolized and conjugated by liver enzymes, we have anticipated that NAFLD would have its metabolic impact on steroid metabolism.

Here, we utilized our previously reported concept, arguing that an individual's urinary steroid metabolite profile represents a subject's unique metabolic fingerprint and offers means of metabolomic phenotyping at the individual level (9,10). Thus, each individual has a unique 'steroidal fingerprint'. A cluster of similar "steroidal fingerprints" related to a disease would be regarded as a 'steroid metabolomic disease signature' (10,11), which represents the impact of a disease in people who differ in their phenotypes or have other health problems. We have previously clustered steroidal fingerprints of children with nonsyndromic obesity into five clusters with distinctive steroidal signatures (11).

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Here, we analyzed the clinical data of a group of 85 patients with well-phenotyped non-syndromic childhood obesity and defined those affected and those unaffected by NAFLD and/or elevated activities of liver enzymes. We generated steroidal disease signatures of the two groups and suggest that it might shed light on steroid-related metabolic sequelae of liver disease in childhood obesity.

Subjects and Methods

Between March 2012 and August 2013, we examined a consecutive series of 117 obese Caucasians children and adolescents (BMI > 97th centile). They were recruited from the patients referred to the Department of Pediatric Endocrinology, Medical University of Silesia, Katowice, Poland. After exclusion of younger participants (<8 years), syndromic obesity, chronic diseases, pharmacotherapy (also metformin) or precocious puberty, we included the remaining 85 patients (43 girls), aged 8.5-18.0 years (mean age 14.4, SD 2.33, median 14.5 years).

All patients underwent a clinical assessment and diagnostic procedures that included general physical examination, anthropometric measurements of height, weight, waist and hip circumference and puberty assessment, as previously described (11). Morning fasting venous blood samples were collected to measure lipids, glucose (GLU), insulin (INS), TSH, fT4, cortisol and aminotransferases. Plasma total cholesterol (T Chol), high-density lipoprotein cholesterol (HDL-Chol) and triglyceride (TG) levels were analyzed enzymatically (Beckman Coulter, USA). GLU and INS levels were also measured in an oral glucose tolerance test (OGTT, 1.75 g/kg, max 75 g). Enzymatic test (hexokinase method) was used for the quantitative determination of glucose (Beckman Coulter, USA). INS was determined using a chemiluminescence immunoassay on Immulite 2000 analyzer (DPC, USA). Fasting

INS/GLU ratio (FIGR) and homeostatic model assessment of INS resistance (R-HOMA, fasting GLU [mmol/L] x fasting INS [mIU/L]/22.5 were calculated as indices of insulin resistance. Cortisol was measured in the morning (8am) and midnight using chemiluminescent immunoassay by Immulite 2000 analyzer (DPC, USA). Serum concentrations of fT4 and TSH were measured with a chemiluminescent immunometric assay (Siemens, Immulite 2000 Free T4 , Immulite 2000 Third Generation TSH, USA). Gamma-glutamyl transpeptidase (GGTP), alanine (ALT/GPT) and aspartate aminotransferases (AST/SGOT) activity in the serum were assessed according to International Federation in Clinical Chemistry (Beckman Coulter, USA).

Assessment of Liver Disease / NAFLD

Abdomen ultrasonography to evaluate the liver for hepatic steatosis features was performed with 5 MHz convex transducer (Logiq 5, GE Healthcare GmbH, Germany), according to standards in pediatric population. (12). The evidence of hepatic steatosis by abdominal ultrasound (hepatic echogenicity increased above the echogenicity of the adjacent right renal cortex and increase in fine echoes of liver parenchyma compared with intrahepatic vessel borders (7,13)), and no causes for secondary hepatic fat accumulation, defined here nonalcoholic fatty liver disease (NAFLD) diagnosis (5,7,12). Any markers of liver dysfunction (elevated ALT >45 U/L – $L1^{A}$, NAFLD based on ultrasonography – $L1^{L}$, or both – $L1^{AL}$) where defined as liver disease – L1 as compared to L0 – without markers of liver disease.

Gas chromatography-mass spectrometry (GC-MS) of urinary steroids

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Steroid metabolites in 24-h-urine samples were analyzed by quantitative targeted GC-MS (9,10,11). Briefly, free and conjugated urinary steroids were extracted by solid phase extraction and conjugates were enzymatically hydrolyzed. After recovery of hydrolyzed steroids by solid phase extraction, known amounts of internal standards (5α-androstane-3α,17α-diol, stigmasterol) were added to each extract before formation of methyloxime-trimethylsilyl ethers. GC was performed using an Optima-1 fused silica column (Macherey–Nagel, Dueren, Germany) housed in an Agilent Technologies 6890 series GC that was directly interfaced to an Agilent Technologies 5975 inert XL mass selective detector. After calibration, values for the excretion of individual steroids were determined by measuring the selected ion peak areas against the internal standard areas.

Steroid metabolites' ratios, as described in our previous paper (11), were used to calculate the activity of the enzymes: 5α reductase (An/Et; 5αTHF/THF, 5αTHB/THB), 11β-hydroxysteroid dehydrogenase type 1 -11βHSD1 ([THF+αTHF]/THE), 3β-hydroxysteroid dehydrogenase- 3βHSD ([THE+THF+αTHF]/P5T-17α) and 21-hydroxylase ([THE+THF+αTHF]/PT, [THE+THF+αTHF]/ PO5α3α)

The study was conducted according to Helsinki declaration, and approved by the Ethics Committee of the Medical University of Silesia. Informed consent was obtained from each patient over age 16, a parent or a legal guardian, after full explanation of the purpose and nature of all procedures.

Statistical analysis and visualization of metabolomic data.

Steroid metabolites quantities were z-transformed based on sex and age-adjusted normal reference groups, as described elsewhere (11). Per each of 31 z-transformed steroid metabolites and per each one of the groups L0, L1 and subgroups $L1^{A}$, $L1^{L}$, $L1^{AL}$ the median was computed. The standard R-function 'matplot' (https://www.R-project.org/) (14) was used to depict the steroidal signatures of each subgroup as the difference between the above medians versus the median of L0 group (11).

Clinical and chemical data as well as steroid metabolites' concentrations ratios of patients in each group were analyzed, and t-student test, t-test with separate variance estimation, ANOVA or Kruskal-Wallis ANOVA where appropriate were utilized to assess the difference between groups. p-value <0.05 was considered statistically significant.

Results

Clinical phenotype

Out of 85 obese children, a liver disease was diagnosed in 21 (21/85, 24.7%; L1); in 5 patients by elevated ALT activity ($L1^{A}$), in 10 by sonographic liver steatosis ($L1^{L}$) and in 6 by both markers ($L1^{AL}$). The clinical phenotype is presented in Tables 1a and 2a.

The mean age, BMI, BMI z-score, hSDS, waist and hip circumference as well as blood pressure values were not significantly different between patients of groups L0 and L1 and among L0, $L1^{A}$, $L1^{L}$, $L1^{AL}$ subgroups. There were relatively more males in L1 group than in L0 (14/21, 67% vs. 28/64, 44%, Table 1a).

Chemical phenotype

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At the biochemical level, patients of L1 comparing to L0 group presented higher concentration of fasting triglycerides and insulin, postprandial glucose and insulin. Both indices of insulin resistance, insulin/glucose ratio (INS/GLU) and HOMA-IR, GGTP, AST/SGOT activity were significantly higher in L1 group. Comparison of three liver affected subgroups $L1^{A}$, $L1^{L}$, $L1^{AL}$ and L0 (ANOVA) confirmed significantly the highest level of T-cholesterol and triglycerides in $L1^{AL}$ patients. Fasting INS and postprandial GLU levels were higher in $L1^{L}$ and $L1^{AL}$ patients than in $L1^{A}$ and L0, postprandial INS was the highest in $L1^{L}$ group. INS/GLU ratio and HOMA-IR values were also the highest in both groups with liver steatosis features in the ultrasonography - $L1^{L}$ and $L1^{AL}$. The highest GGTP and AST/SGOT activities were observed in $L1^{A}$ group (Table 2b).

Steroidal signature of liver disease

Obese patients of the L0 group presented higher midnight plasma cortisol concentration ($p<0.001$) than the L1 group (Table 1b). Comparing the z-transformed values of steroid metabolites, significantly higher tetrahydrocortisone (THE) concentration were found in L1 group ($p=0.046$).

'Steroidal signature' of liver disease is presented as the difference between z-transformed concentrations of steroid metabolites in L0 and L1 patients (Figure 1). Liver-affected patients have shown significantly enhanced 5α-reductase and 21-hydroxylase activity and lower activity of 11βHSD1 than L0 subjects (Table 3).

'Steroid metabolomic disease signature' of $L1^{A}$, $L1^{L}$, $L1^{AL}$ are presented in Figure 2 a, b &c). Liver affected patients $L1^{L}$ presented significantly enhanced activity of 21-hydroxylase, and those with elevated ALT ($L1^{A}$ & $L1^{AL}$) showed enhanced 5α-reductase activity (Table 4).

Discussion

Based on our previous definition of 'steroid metabolomic disease signature' by quantitative urinary steroidal GC-MS data (10,11), here we define the steroidal signature of liver disease in non-syndromic childhood obesity.

The results emphasize the fact that the clinical picture of obese children with liver disease is not different from that of obese children with no liver disease; they have comparable age, height, weight, BMI, waist and hip circumference and blood pressure. They have comparable serum TSH and 8 am cortisol, while their midnight cortisol is lower. Those with liver disease have higher circulating triglycerides, though their lipoproteins are comparable, as previously reported (15). We confirm that obesity and insulin resistance play important roles in the development of NAFLD (16). The insulin sensitivity indices of obese children with liver disease are marked by higher postprandial glucose and insulin, higher insulin/glucose ratio and higher HOMA-IR (17) than those with no liver disease.

This complex 'steroidal signature' of liver disease reflects previously published single observations. The steroidal disease signature is marked by low urinary DHEA (18,19) and its metabolites, higher glucocorticoid metabolites, due to increased glucocorticoid production rate (20), and lower mineralocorticoid metabolites. It is characterized by derangement of the cortisol/cortisone shuttle generated by 11β hydroxysteroid dehydrogenase (HSD) type 1 (20), as is evident from the lower (THF+αTHF)/THE ratio, enhanced 3βHSD activity ([THE+THF+αTHF]/5PT-17α ratio) and enhanced 21-hydroxylase activity ([THE+THF+αTHF]/PT). These findings may suggest lesser hepatic recycling (reduction) of cortisone to cortisol in liver steatosis, which is compensated for by increased adrenal cortisol generation and further metabolic consequences resulting from higher glucocorticoids concentrations - this mechanism resulting in a model of a vicious circle.

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Therefore, it is not surprising that higher tetrahydrocortisone concentration in L1 patients corresponds with unfavorable biochemical profile: higher triglycerides and insulin resistance. The clinical profile, however, defined by BMI z-score or waist circumference, is not useful in the prediction of liver disease as well as other obesity complications.

A previous study focused on the measurement of circulating DHEAS, and found low DHEAS in NASH patient. The authors assumed that this might have resulted from reduced sulphonation of DHEA (19). Low sulphonation of steroids has been also found in a study in obese children (21). A further important feature of the obesity-associated liver disease signature is the low urinary DHEA excretion rate and its metabolites. It was previously suggested that DHEA treatment reduced hepatic injury in experimental animals by inhibiting several inflammatory mediators such as tumor necrosis factor-α and macrophage mitogen inhibitory factor, and preventing the increase in serum ALT levels (22). Thus, we speculate that DHEA might have a protective effect against hepatotoxicity. It has been shown that DHEA inhibits 11β-hydroxysteroid dehydrogenase -1 expression in liver and adipose tissues (23) - another component of the steroidal signature. The liver is also the site of greatest activity of 11βHSD (24), and as such responds to liver disease with decreasing activity. Obesity *per se* tends to enhance 11βHSD-1 activity (25), but insulin resistance, a prominent manifestation of the metabolic syndrome in obesity and the group of children presented here, inhibits 11βHSD-1 activity (26). Moreover, insulin resistance and the metabolic syndrome are involved in the development and progression of NAFLD (15).

In conclusion, we present the 'disease signature" of liver disease in childhood obesity. We are aware of the limitations of our study as our results may be biased by observational cross-sectional character of the study and the relatively small number of participants in subgroups with liver dysfunction. Moreover, we did not quantify the ultrasonographic steatosis, other than visually. However, our findings suggest a new approach to the diagnosis and further

242 understanding of the metabolic consequences of liver disease as part of the metabolic
243 syndrome of obesity. They reflect the derangements of steroid metabolism in NAFLD that
244 includes enhanced glucocorticoid production and deranged androgens and
245 mineralocorticoids, and suggests a protective effect of DHEA on the liver in childhood
246 obesity. Knowledge of these sequels may provide ways for personalized medicine in obese
247 children with liver disease. Future prospective intervention study is also needed to verify if
248 obtained findings are only reversible consequences of obesity or whether they reflect non-
249 modifiable individual genetic predisposition.

250

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257 **Author contribution statement**

258 Conceptualization, AG, ZH, MS, MFH and SAW.; Methodology, ZH, AG, MS, MFH, SAW,
259 ZO and KG.; Software, MS and AG.; Validation, AG and MS.; Formal Analysis, MS;
260 Investigation, AG.; Resources AG and SAW; Data Curation, AG.; Writing – Original Draft
261 Preparation, ZH, AG, MS, MFH and SAW.; Writing – Review & Editing, ZH, AG, MS,
262 MFH, SAW, ZO, KG.; Visualization, ZO and KG.; Supervision, ZH and AG.; Project
263 Administration, AG.; Funding Acquisition, AG.

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References

1. Molleston JP, White F, Teckman J & Fitzgerald JF. Obese children with steatohepatitis can develop cirrhosis in childhood. *Am J Gastroenterol* 2002 **97** 2460-2462. doi: 10.1111/j.1572-0241.2002.06003.x

2. Marchesini G, Brizi M, Bianchi G, Tomassetti S, Bugianesi E, Lenzi M, McCullough AJ, Natale S, Forlani G & Melchionda N. Nonalcoholic fatty liver disease a feature of the metabolic syndrome. *Diabetes* 2001 **50** 1844-1850. doi.org/10.2337/diabetes.50.8.1844

3. Temple JL, Cordero P, Li J, Nguyen V & Oben JA. A Guide to Non-Alcoholic Fatty Liver Disease in Childhood and Adolescence. *Int J Mol Sci* 2016 **17**(6). pii: E947. doi: 10.3390/ijms17060947.

4. European Association for the Study of the Liver (EASL); European Association for the Study of Diabetes (EASD); European Association for the Study of Obesity (EASO).EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. *J Hepatol* 2016 **64** 1388-402. doi: 10.1016/j.jhep.2015.11.004.

5. Chalasani N, Younossi Z, Lavine JE, Diehl AM, Brunt EM, Cusi K, Charlton M & Sanyal AJ. The diagnosis and management of non-alcoholic fatty liver disease: Practice Guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association. *Hepatology* 2012 **55** 2005-2023, doi: 10.1002/hep.25762

6. Shannon A, Alkhouri N, Carter-Kent C, Monti L, Devito R, Lopez R, Feldstein AE & Nobili V. Ultrasonographic quantitative estimation of hepatic steatosis in children With NAFLD. *J Pediatr Gastroenterol Nutr* 2011 **53**(2) 190-5, doi: 10.1097/MPG.0b013e31821b4b61.

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294 7. Vajro P, Lenta S, Socha P, Dhawan A, McKiernan P, Baumann U, Durmaz O, Lacaille F,
295 McLin V & Nobili V. Diagnosis of nonalcoholic fatty liver disease in children and
296 adolescents: position paper of the ESPGHAN Hepatology Committee. *J Pediatr*
297 *Gastroenterol Nutr* 2012 **54**(5) 700-13. doi: 10.1097/MPG.0b013e318252a13f.
298 8. Thomsen KL, Grønbæk H, Glavind E, Hebbard L, Jessen N, Clouston A, George J &
299 Vilstruo H. Experimental nonalcoholic steatohepatitis compromises ureagenesis, an
300 essential hepatic metabolic function. *Am J Physiol Gastrointest Liver Physiol* 2014
301 **307** G295-G301. doi: 10.1152/ajpgi.00036.2014.
302 9. Wudy SA & Hartmann MF. Gas chromatography-mass spectrometry profiling of steroids
303 in times of molecular biology. *Horm Metab Res* 2004 **36** 415-422. DOI: 10.1055/s-
304 2004-814565
305 10. Vitkin E, Ben-Dor A, Shmoish M, Hartmann MF, Yakhini Z, Wudy SA, & Hochberg Z.
306 Peer group normalization and urine to blood context in steroid metabolomics: the case
307 of CAH and obesity. *Steroids* 2014 **88** 83-89. doi: 10.1016/j.steroids.2014.07.003.
308 11. Gawlik A, Shmoish M, Hartmann MF, Malecka-Tendera E, Wudy SA & Hochberg Z.
309 Steroid Metabolomic Disease Signature of Nonsyndromic Childhood Obesity. *J Clin*
310 *Endocrinol Metab* 2016 **101** 4329-4337. DOI: 10.1210/jc.2016-1754
311 12. Brzewski M. Current standards in abdominal cavity ultrasound examination in children. *J*
312 *Ultrason* 2017 **17**(68) 41-42. doi: 10.15557/JoU.2017.0006.
313 13. Borges VF, Diniz AL, Cotrim HP, Rocha HL, Andrade NB. Sonographic hepatorenal
314 ratio: a noninvasive method to diagnose nonalcoholic steatosis. *J Clin Ultrasound*
315 2013 **41** 18-25. doi: 10.1002/jcu.21994.
316 14. R Core Team. R: A language and environment for statistical computing. R Foundation for
317 Statistical Computing, Vienna, Austria. 2017 URL https://www.R-project.org/.

15. Pacifico L, Bonci E, Andreoli G, Romaggioli S, Di Miscio R, Lombardo CV & Chiesa C. Association of serum triglyceride-to-HDL cholesterol ratio with carotid artery intima-media thickness, insulin resistance and nonalcoholic fatty liver disease in children and adolescents. *Nutr Metab Cardiovasc Dis* 2014 **24** 737-743. doi: 10.1016/j.numecd.2014.01.010.

16. Sanyal AJ, Campbell–Sargent C, Mirshahi F, Rizzo WB, Contos MJ, Sterling RK, Luketic VA, Shiffman ML & Clore JN. Nonalcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. *Gastroenterology* 2001 **120** 1183-1192. DOI: 10.1053/gast.2001.23256

17. Ozhan B, Ersoy B, Kiremitci S, Ozkol M & Taneli F. Insulin sensitivity indices: fasting versus glucose-stimulated ındices in pediatric non-alcoholic fatty liver disease. *Eur Rev Med Pharmacol Sci* 2015 **19** 3450-3458.

18. Charlton M, Angulo P, Chalasani N, Merriman R, Viker K, Charatcharoenwitthaya P, Sanderson S, Gawrieh S, Krishnan A & Lindor K. Low circulating levels of dehydroepiandrosterone in histologically advanced nonalcoholic fatty liver disease. *Hepatology* 2008 **47** 484-492. doi: 10.1002/hep.22063.

19. Koga M, Saito H, Mukai M, Saibara T & Kasayama S. Serum dehydroepiandrosterone sulphate levels in patients with non-alcoholic fatty liver disease. *Intern Med* 2011 **50** 1657-1661. DOI: https://doi.org/10.2169/internalmedicine.50.4682

20. Ahmed A, Rabbitt E, Brady T, Brown C, Guest P, Bujalska IJ, Doig C, Newsome PN, Hubscher S, Elias E *et al*. A switch in hepatic cortisol metabolism across the spectrum of non alcoholic fatty liver disease. *PLoS One* 2012 **7** e29531. doi: 10.1371/journal.pone.0029531.

Accepted Manuscript published as EC-18-0536.R2. Accepted for publication: 09-May-2019

341 21. Reinehr T, Sánchez-Guijo A, Lass N & Wudy SA. Higher steroid sulfation is linked to
342 successful weight loss in obese children. *Endocr Connect* 2018 **7**(10) 1020–1030. doi:
343 10.1530/EC-18-0233
344 22. Yoneda M, Wada K, Katayama K, Nakajima N, Iwasaki T, Osawa E, Mukasa K, Yamada
345 Y, Blumberg RS, Sekihara H & Nakajima A. A novel therapy for acute hepatitis
346 utilizing dehydroepiandrosterone in the murine model of hepatitis. *Biochem*
347 *Pharmacol* 2004 **68** 2283-2289. DOI: 10.1016/j.bcp.2004.07.044
348 23. Apostolova G, Schweizer RA, Balazs Z, Kostadinova RM & Odermatt A.
349 Dehydroepiandrosterone inhibits the amplification of glucocorticoid action in adipose
350 tissue. *Am J Physiol-Endocrinol Metab* 2005 **288** E957-E964. DOI:
351 10.1152/ajpendo.00442.2004
352 24. Gathercole LL, Lavery GG, Morgan SA, Cooper MS, Sinclair AJ, Tomlinson JW &
353 Stewart PM. 11β-Hydroxysteroid dehydrogenase 1: translational and therapeutic
354 aspects. *Endocr Rev* 2013 **34** 525-555. doi: 10.1210/er.2012-1050
355 25. Tiosano D, Eisentein I, Militianu D, Chrousos GP & Hochberg Ze. 11β-hydroxysteroid
356 dehydrogenase activity in hypothalamic obesity. *J Clin Endocrinol Metab* 2003 **88**
357 379-384. DOI: 10.1210/jc.2002-020511
358 26. Liu Y-J, Nakagawa Y, Nasuda K, Saegusa H & Igarashi Y. Effect of growth hormone,
359 insulin and dexamethasone on 11 β-hydroxysteroid dehydrogenase activity on a
360 primary culture of rat hepatocytes. *Life Sci* 1996 **59** 227-234. DOI:
361 https://doi.org/10.1016/0024-3205(96)00288-3

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363

365 **Figure legends**

366 Figure 1. Steroidal signature of liver disease in childhood obesity: differences between z-
367 transformed concentrations of steroid metabolites (androgens, glucocorticoids and
368 mineralocorticoids) in liver diseases (L1) and with non-liver disease features (L0) patients.

369

370 Figure 2 Steroidal signatures of liver disease in childhood obesity: differences between z-
371 transformed concentrations of steroid metabolites (androgens, glucocorticoids and
372 mineralocorticoids) in non-liver disease features (L0) patients and (a) patients with ALT+
373 ($L1^{A}$), (b) patients with sonographic liver steatosis ($L1^{L}$) and (c) patients with ALT+ and
374 sonographic liver steatosis ($L1^{AL}$)

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Table 1 Comparison of clinical (a) and chemical (b) phenotype of non-liver disease patients (L0) and patients with liver disease features (L1)

Legend:; L0 – non-liver disease patients. L1- liver disease patients (ALT+ or sonographic liver steatosis or both). f-female; m – male; hSDS-height standard deviation score; BMI – body mass index; WHR- waist to hip ratio; BP – blood pressure; TSH - thyroid-stimulating hormone; TChol – total cholesterol; HDL-Chol – HDL-cholesterol; TG- triglicerydes; GLU- glucose; INS- insulin; ALT/GPT -alanine aminotransferases; AST/SGOT - aspartate aminotransferases; GGTP - Gamma-glutamyl transpeptidase; NS- not significant. Values are means and SD; Significance by t-student test.

	Mean L0 (n=64)	**SD L0**	**Mean L1 (n=21)**	**SD L1**	***p-value***
a. Clinical phenotype					
sex [f/m]	36/28		7/14		*0.001*
age [years]	14.4	2.3	14.0	2.4	NS
weight [kg]	87.0	20.0	91.2	21.8	NS
height [cm]	164.0	11.4	166.1	11.5	NS
hSDS	0.4	1.4	0.8	1.6	NS
BMI [kg/m^2]	32.0	5.2	32.8	5.4	NS

BMI- z score IOTF	2.7	0.5	2.8	0.5	NS
waist [cm]	100.6	11.5	102.1	11.6	NS
WHR	0.9	0.1	1.0	0.0	*0.022*
BP systolic [mm Hg]	125	11	130	12	NS
BP diastolic [mm Hg]	78	8	76	10	NS

	Mean L0 (n=64)	**SD L0**	**Mean L1 (n=21)**	**SD L1**	***p-value***
b. Chemical phenotype					
TSH [µIU/ml]	2.7	1.2	3.0	1.3	NS
cortisol 8.00 [µg/dl]	17.4	6.2	19.7	6.1	NS
cortisol 24.00 [µg/dl]	3.4	3.9	1.6	1.0	*<0.001*
T Chol [mg/dl]	171	35	178	31	NS
HDL-Chol [mg/dl]	50	10	46	12	NS
TG [mg/dl]	134	60	182	76	*0.004*
GLU 0' [mg/dl]	90	9	92	10	NS

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GLU 120'[mg/dl]	112	20	129	21	*0.001*
INS 0' [µIU/ml]	16.7	9.2	35.1	24.1	*0.002*
INS 120' [µIU/ml]	91.6	67.9	163.6	114.2	*0.011*
INS/GLU 0'	0.2	0.1	0.4	0.3	*0.003*
INS/GLU 0' [%>0.3]	10/64, 15.6%		10/21, 47.6%		*0.004*
HOMA-IR	3.7	2.2	8.1	5.6	*0.002*
ALT/GPT [U/l]	24	9	54	29	*<0.001*
AST/SGOT [U/l]	25	8	36	13	*0.002*
GGTP [U/l]	22	10	33	12	*0.006*

Table 2 Comparison of clinical (a) and chemical (b) phenotype of non-liver disease patients (L0) and patients with liver disease features: $L1^{A}$ (ALT+). $L1^{L}$ (sonographic liver steatosis) and $L1^{LA}$ (both ALT+ and sonographic liver steatosis).

Legend: L0 – non-liver disease patients. L1- liver disease patients. $L1^{A}$ – patients L1 with ALT+. $L1^{L}$ – patients L1 with sonographic liver steatosis. $L1^{AL}$ – patients with ALT+ and sonographic liver steatosis); f- female; m-male; hSDS- height standard deviation score; BMI – body mass index; WHR- waist to hip ratio; BP – blood pressure; TSH - thyroid-stimulating hormone; TChol – total cholesterol; HDL-Chol – HDL-cholesterol; TG- triglicerydes; GLU- glucose; INS- insulin; ALT/GPT -alanine aminotransferases; AST/SGOT - aspartate aminotransferases; GGTP - Gamma-glutamyl transpeptidase; NS- not significant. Values are means and SD, Significance by ANOVA.

a. Clinical phenotype	**L0 Mean ±SD (n=64)**	**$L1^{A}$ Mean ±SD (n=5)**	**$L1^{L}$ Mean ±SD (n=10)**	**$L1^{LA}$ Mean ±SD (n=6)**	***p-value***	**All**
sex [f/m]	36/28	2/3	4/6	1/5		43/42
age [years]	14.4±2.3	15.4±1.5	13.0±2.3	14.6±2.6	NS	14.3±2.3
weight [kg]	87.0±20.0	110.3±21.6	81.0±11.9	92.3±26.5	NS	88.1±20.4
height [cm]	164.0±11.4	171.2±5.9	162.6±7.7	167.6±18.4	NS	164.5±11.4
hSDS	0.4±1.4	0.4±0.5	1.2±1.2	0.5±2.6	NS	0.5±1.5
BMI [kg/m^2]	32.0±5.2	37.5±5.9	30.6±3.8	32.4±5.5	NS	32.2±5.2
BMI- z score IOTF	2.7±0.5	3.1±0.5	2.7±0.5	2.8±0.4	NS	2.7±0.5
waist [cm]	100.6±11.5	115.8±16.3	98.6±7.1	98.6±10.1	NS	101.0±11.5
WHR	0.93±0.1	0.98±0.00	0.97±0.0	1.00±0.03	NS	0.95±0.09

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BP systolic [mmHg]	125±11	136±15	129±11	124±5	NS	126±11
BP diastolic [mmHg]	78±8	80±10	76±11	74±5	NS	77±8
b. Chemical phenotype						
TSH [µIU/ml]	2.7±1.2	3.3±1.0	3.0±1.6	2.8±1.1	NS	2.8±1.2
cortisol 8.00 [µg/dl]	17.4±6.1	22.3±3.2	19.3±4.0	18.2±10.0	NS	18.0±6.2
cortisol 24.00 [µg/dl]	3.4±3.9	1.7±1.2	1.5±1.2	1.4±0.2	NS	2.9±3.5
T-Chol [mg/dl]	171±35	182±32	158±18	208±23	*0.029* $L1^{LA}>L1^{L}$	172±34
HDL-chol [mg/dl]	50±10	49±19	43±9	49±10	NS	49±10
TG [mg/dl]	134±60	176±86	184±67	184±94	*0.044* $L1^{LA}$ & $L1^{L}>L0$	146±67
GLU 0' [mg/dl]	90±9	89±9	90±7	97±13	NS	90±9
GLU 120' [mg/dl]	112±20	126±5	130±22	129±30	*0.017* $L1^{L}>L0$	116±21
INS 0' [µIU/ml]	16.7±9.2	23.1±8.7	39.1±29.9	38.5±21.6	*0.001* $L1^{L}$, $L1^{LA}> L0$	21.3±16.3
INS 120' [µIU/ml]	91.6±67.9	139.6±85.4	186.1±140.5	146.0±93.9	*0.033* $L1^{L}>L0$	109.6±87.0
INS/GLU	0.2±0.1	0.3±0.1	0.4±0.3	0.4±0.2	*0.001*	0.2±0.2

					$L1^{L}$, $L1^{LA}$>L0	
INS/GLU [n, %>0.3]	10/64, 15.6%	1/5, 20%	6/10, 60%	3/6, 50%	*0.014*	20/85, 23.5%
					$L1^{L}$, $L1^{LA}$>L0	
HOMA-IR	3.7±2.2	5.1±2.2	8.8±6.7	9.4±5.5	*0.001*	4.8±3.9
					$L1^{LA}$, $L1^{L}$> L0	
ALT/GPT [U/l]	24±9	77±33	34±9	69±28	*<0.0001*	31±21
					$L1^{A}$,$L1^{LA}$>$L1^{L}$>L0	
AST/SGOT [U/l]	25±8	47±14	27±4	43±15	*0.0001*	28±11
					$L1^{A}$,$L1^{LA}$>$L1^{L}$, L0	
GGTP [U/l]	22±10	39±10	26±12	35±12	*0.003*	25±12
					$L1^{A}$, $L1^{LA}$>L0, $L1^{L}$	

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Table 3 Ratio of steroid metabolites (enzyme activity): differences between patients with non-liver diseases (L0) and with liver disease features (L1) patients.

Legend: Values are means and SD; ; L0 – non-liver disease patients. L1- liver disease patients (ALT+ or sonographic liver steatosis or both). NS- not significant. Significance by t-student test.

**Ratio of steroid metabolites was calculated based on steroid metabolites concentrations.*

	Mean L0 (n=64)	**SD L0**	**Mean L1 (n=21)**	**SD L1**	***p-value***
An/Et (5α reductase)	2.1	0.9	2.5	0.9	*0.041*
5αTHF/THF (5α reductase)	1.3	0.6	1.3	0.5	NS
5αTHB/THB (5α reductase)	3.4	1.8	3.3	1.3	NS
(THF+αTHF)/THE (11βHSD1)	0.9	0.3	0.7	0.2	*0.019*
(THE+THF+αTHF)/P5T-17α (3βHSD)	19.1	15.4	24.2	21.3	NS
(THE+THF+αTHF)/PT (21-OHase)	10.1	3.9	13.6	5.1	*0.009*
(THE+THF+αTHF)/PO5α3α (21-OHase)	300.9	160.4	320.7	193.3	NS

Table 4 Ratio of steroid metabolites (enzyme activity): differences between non-liver disease patients (L0) and patients with liver disease features: $L1^{A}$ (ALT+). $L1^{L}$ (sonographic liver steatosis) and $L1^{LA}$ (both ALT+ and sonographic liver steatosis).

Legend: Values are means and SD; L0 – non-liver disease patients. L1- liver disease patients. $L1^{A}$ – patients L1 with ALT+. $L1^{L}$ – patients L1 with sonographic liver steatosis. $L1^{AL}$ – patients with ALT+ and sonographic liver steatosis. NS- not significant. Significance by ANOVA.

**Ratio of steroid metabolites was calculated based on steroid metabolites concentrations.*

Ratio	L0 Mean ±SD (n=64)	$L1^{A}$ Mean ±SD (n=5)	$L1^{L}$ Mean ±SD (n=10)	$L1^{LA}$ Mean ±SD (n=6)	*p-value*	All
An/Et (5α-reductase)	2.1±0.9	2.9±1.1	2.0±0.7	3.1±0.8	*0.011* $L1^{LA}$, $L1^{A}>L0, L1^{L}$	2.2±0.9
5αTHF/THF (5α-reductase)	1.3±0.6	1.4±0.4	1.1±0.4	1.7±0.5	NS	1.3±0.5
5αTHB/THB (5α-reductase)	3.4±1.8	3.6±0.8	2.7±1.1	4.0±1.6	NS	3.4±1.7
(THF+αTHF)/THE (11βHSD1)	0.9±0.3	0.8±0.1	0.7±0.2	0.8±0.2	NS	0.8±0.3
(THE+THF+αTHF)/P5T-17α (3βHSD)	19.1±15.4	15.1±8.1	27.5±17.1	26.4±33.5	NS	20.4±17.0
(THE+THF+αTHF)/PT (21-OH)	10.1±3.9	12.8±6.0	14.4±4.6	12.7±5.9	*0.016* $L1^{L}>L0$	11.0±4.5
(THE+THF+αTHF)/PO5α3α (21-OH)	300.9±160.4	228.5±99.0	416.1±230.8	238.4±104.0	NS	305.8±168.1

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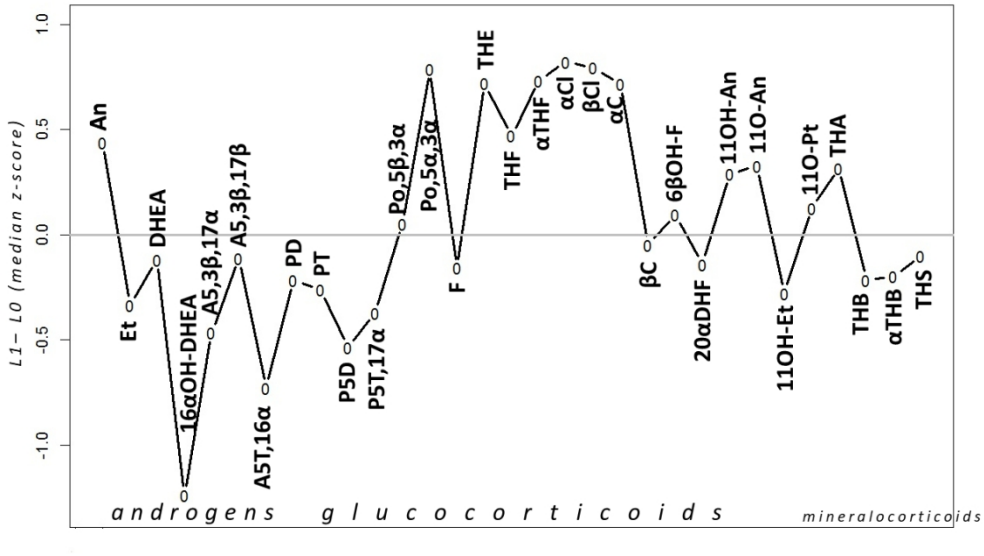

Figure 1. Steroidal signature of liver disease in childhood obesity: differences between z-transformed concentrations of steroid metabolites (androgens, glucocorticoids and mineralocorticoids) in liver diseases (L1) and with non-liver disease features (L0) patients.

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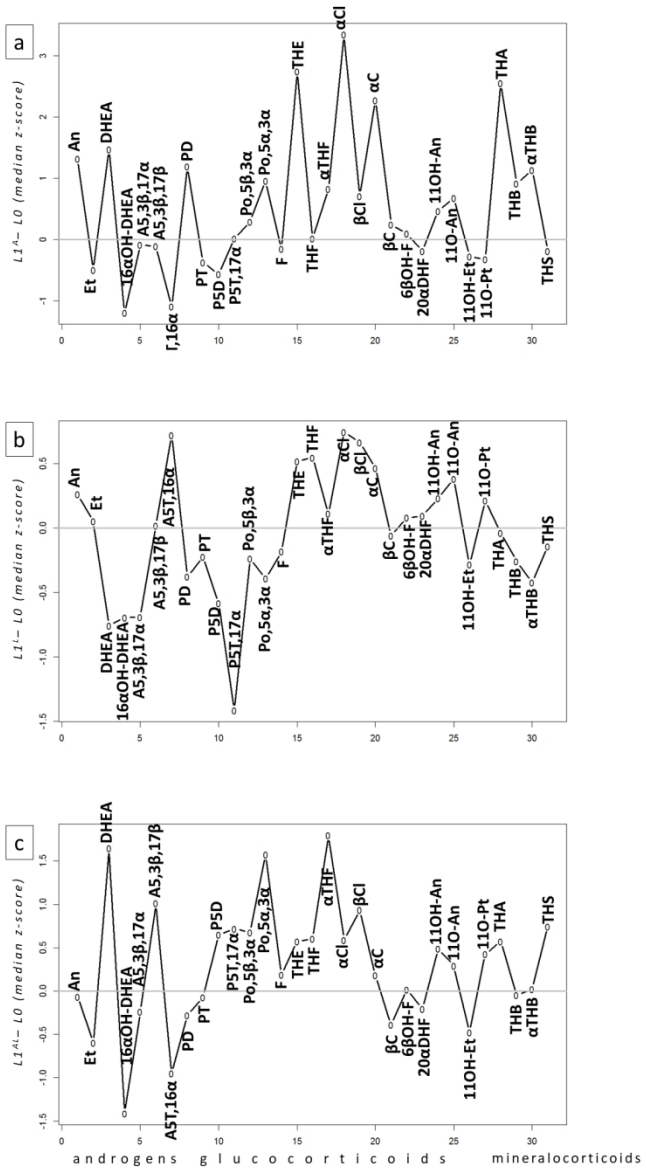

Figure 2 Steroidal signatures of liver disease in childhood obesity: differences between z-transformed concentrations of steroid metabolites (androgens, glucocorticoids and mineralocorticoids) in non-liver disease features (L0) patients and (a) patients with ALT+ (L1A), (b) patients with sonographic liver steatosis (L1L) and (c) patients with ALT+ and sonographic liver steatosis (L1AL)

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