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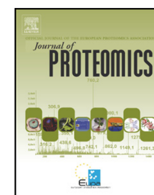

# Liver lipid metabolism is altered by increased circulating estrogen to androgen ratio in male mouse

Anni P. Vehmas [a,b], Marion Adam [b,c], Teemu D. Laajala [c,d,e,f], Gabi Kastenmüller [g], Cornelia Prehn [h], Jan Rozman [i,j,k], Claes Ohlsson [l], Helmut Fuchs [i], Martin Hrabě de Angelis [i,j,m], Valérie Gailus-Durner [i], Laura L. Elo [a,d], Tero Aittokallio [d,f], Jerzy Adamski [h,j,m], Garry Corthals [a,n], Matti Poutanen [b,c,o], Leena Strauss [b,c,*]

[a] Turku Centre for Biotechnology, University of Turku and Åbo Akademi University, Turku, Finland
[b] Department of Physiology, Institute of Biomedicine, University of Turku, Turku, Finland
[c] Turku Center for Disease Modeling, University of Turku, Turku, Finland
[d] Department of Mathematics and Statistics, University of Turku, Turku, Finland
[e] Drug Research Doctoral Programme, University of Turku, Finland
[f] Institute for Molecular Medicine Finland (FIMM), University of Helsinki, Finland
[g] Institute of Bioinformatics and Systems Biology, Helmholtz Zentrum München, Neuherberg, Germany
[h] Genome Analysis Center, Institute of Experimental Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, Neuherberg, Germany
[i] German Mouse Clinic, Institute of Experimental Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, Neuherberg, Germany
[j] German Center for Diabetes Research (DZD), Neuherberg, Germany
[k] Molecular Nutritional Medicine, Else Kröner-Fresenius Center, Technische Universität München, Freising-Weihenstephan, Germany
[l] Centre for Bone and Arthritis Research, Institute of Medicine, University of Gothenburg, Gothenburg, Sweden
[m] Experimental Genetics, Center of Life and Food Sciences Weihenstephan, Technische Universität München, Neuherberg, Germany
[n] Van 't Hoff Institute for Molecular Sciences, University of Amsterdam, The Netherlands
[o] Institute of Medicine, Sahlgrenska Academy, University of Gothenburg, Sweden

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ABSTRACT

Estrogens are suggested to lower the risk of developing metabolic syndrome in both sexes. In this study, we investigated how the increased circulating estrogen-to-androgen ratio (E/A) alters liver lipid metabolism in males. The cytochrome P450 aromatase (P450arom) is an enzyme converting androgens to estrogens. Male mice overexpressing human aromatase enzyme (AROM+ mice), and thus have high circulating E/A, were used as a model in this study. Proteomics and gene expression analyses indicated an increase in the peroxisomal β-oxidation in the liver of AROM+ mice as compared with their wild type littermates. Correspondingly, metabolomic analysis revealed a decrease in the amount of phosphatidylcholines with long-chain fatty acids in the plasma. With interest we noted that the expression of Cyp4a12a enzyme, which specifically metabolizes arachidonic acid (AA) to 20-hydroxy AA, was dramatically decreased in the AROM+ liver. As a consequence, increased amounts of phospholipids having AA as a fatty acid tail were detected in the plasma of the AROM+ mice. Overall, these observations demonstrate that high circulating E/A in males is linked to indicators of higher peroxisomal β-oxidation and lower AA metabolism in the liver. Furthermore, the plasma phospholipid profile reflects the changes in the liver lipid metabolism.

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## 1. Introduction

Estrogens play an important role in male physiology and pathophysiology. The final step in the biosynthesis of estrogens from androgens is catalyzed by the cytochrome P450 aromatase (P450arom). Men with a non-functional aromatase enzyme, and consequently undetectable levels of circulating estrogens, are reported to have tall stature with delayed epiphyseal closure, osteoporosis and impaired reproductive functions [1–3]. In addition, they suffer from metabolic disorder-related symptoms such as dyslipidemia, obesity and insulin resistance, and some of the men have been reported to have liver steatosis [4]. Consistent with human studies, liver steatosis connected to increased circulating triglyceride and cholesterol levels have been detected in aromatase deficient mice (ArKO mice). Dyslipidemia in ArKO mice has been explained by increased lipid biosynthesis, decreased fatty acid β-oxidation and glucose intolerance in the liver [5–7]. The liver phenotype has been shown to normalize after estrogen treatment in both aromatase deficient men and ArKO mice [4,5],

We have previously reported that male mice universally expressing human aromatase enzyme (AROM+ mice), and thus having increased

* Corresponding author at: Department of Physiology, Institute of Biomedicine, University of Turku, FI-20014 Turku, Finland.
*E-mail address:* leesal@utu.fi (L. Strauss).

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conversion of androgens to estrogens, are feminized. Although female mice have no phenotype, the male AROM+ mice present with gynecomastia and severe disorders in their reproductive organs [8,9]. The hormonal levels and consequently the feminized phenotype in the AROM+ males are normalized after aromatase inhibitor treatment [10]. Thus, the AROM+ mouse model is a suitable tool to study the consequences of increased estrogen exposure in males in the context of reduced levels of androgens.

The aim of the present study was to understand how an increase in E/A regulates liver lipid metabolism in males. In this work, the effect of high circulating estrogen-to-androgen ratio (E/A) on liver proteome and transcriptome, and consequently on plasma phospholipid profile, was studied by a combination of LC–MS label free proteomics, mRNA microarray and phospholipid profiling by LC–MS/MS. We show that high E/A in male mice is linked to higher peroxisomal β-oxidation and lower arachidonic acid (AA) metabolism in the liver, and consequently to altered plasma phospholipid profile. In this context it is also interesting to note that altered plasma phospholipid profile has been recently linked to several diseases, such as hepatocellular carcinoma and liver cirrhosis [11] and type 1 diabetes [12]. The results of this study demonstrate that plasma phospholipid profiling provides specific information on lipid metabolism in the liver and could be used as a marker for liver steatosis and other metabolic disorders.

## 2. Materials and methods

### 2.1. Mouse model

The AROM+ transgenic mouse model has been described previously [8]. Mice aged between 14 and 18 weeks were used for the proteomics, transcriptomics and metabolomics analyses. The mice were given soy-free natural ingredient feed (Special Diets Services, Witham, UK) and tap water ad libitum, and housed in specific pathogen-free conditions at Central Animal Laboratory, University of Turku, complying with international guidelines on the care and use of laboratory animals. Animal handling was conducted in accordance with Finnish Animal Ethics Committee and the institutional animal care policies of the University of Turku (Turku, Finland), which fully meet the requirements as defined in the NIH Guide on animal experimentation (NIH publication 86-23). For screening the general bioenergetics parameters, animals were shipped to the German Mouse Clinic (GMC) and maintained in IVC cages according to GMC housing conditions and German laws. All tests performed at the GMC were approved by the responsible authority of the government of Upper Bavaria. The GMC screen comprises extensive, standardized phenotyping of mice between the age of 9 and 21 weeks [13]. To confirm the altered sex steroid concentrations between AROM+ and wild type (WT) mice serum, the concentrations of estradiol (E2), estrone (E1), testosterone (T) and androstenedione (A-dione) were analyzed in a single run by validated gas chromatography tandem mass spectrometry method [14]. The analyzed mice were at the age of 2 months.

### 2.2. Proteomics

Liver samples of seven WT and five AROM+ male mice were prepared as described previously by Kanerva and coworkers [15] with minor modifications: The samples were homogenized in the presence of complete Mini EDTA-free Protease Inhibitor Cocktail (Roche, Basel, Switzerland) and the protein concentration was measured by DC Assay (Biorad). The proteins were precipitated by acetone and subjected to trypsin digestion with the trypsin–protein ratio of 1/60. The samples were analyzed on an LTQ Orbitrap Velos Pro mass spectrometer coupled to an EASY-nLC liquid chromatography system (Thermo Scientific). Sample loading, solvent delivery and scan functions were controlled by Xcalibur software (v2.1.0 SP1.1160; Thermo Scientific).

The 12 mouse liver peptide samples were injected in a randomized order and a combined sample consisting of equal amount of peptides from each liver sample was injected four times at regular intervals (Fig. 1). An amount of 200 ng of peptides, as estimated by the measurement of the absorbance at 280 nm by Nanodrop ND-1000 spectrophotometer (version 3.7.1, Thermo Fisher Scientific), was used in each injection. Peptide elution was accomplished by a 95 min long gradient from 98% solvent A (98% $H_2O$, 2% ACN and 0.2% HCOOH) to 35% solvent B (95% ACN, 5% $H_2O$ and 0.2% HCOOH) with a flow rate 0.3 μl/min. Peptides were subjected to reversed-phase separation by a 2.5 cm long, 75 μm inner diameter trap column, and a 15 cm long, 75 μm inner diameter, analytical column packed in-house with C18 particles (Magic AQ C18 resin — 5 μm/200 Å, Bruker-Michrom, Billerica, MA, USA).

The Orbitrap mass analyzer was operated in a positive-ion mode in a mass range of 300–2000 m/z. A preview scan followed by a survey scan (MS1) at a resolution of 60,000 was executed in each cycle. Precursor ions were selected for fragmentation (MS/MS) by collision induced dissociation (CID) in the ion trap mass analyzer, after which they were added to an exclusion list for 60 s. The 12 mouse liver peptide samples and the first injection of the combined sample were analyzed in Orbitrap Velos Pro in Data Dependent Acquisition (DDA) mode, where the 15 most intense doubly or triply charged parent masses were automatically selected for fragmentation (Fig. 1).

Combined sample was injected at regular intervals to monitor the technical performance of the liquid chromatography and MS1-level quantification throughout the sample series. However, to minimize the redundancy in the precursor ion identifications (MS/MS-level) for the combined sample, a directed proteomics approach was chosen [16,17]. Therefore, the injections 2–4 of the combined sample were analyzed by only fragmenting the precursors that were not identified in the previous injections of the sample (Fig. 1). This was accomplished by the construction of inclusion lists consisting of unidentified and unfragmented precursor ion masses by Progenesis 4.0 (Nonlinear Dynamics, Newcastle upon Tyne, UK). In Progenesis the precursor ions were recognized by an automatic feature detection algorithm. An inclusion list was constructed containing those precursors (2+, 3+) that were eluted in a window of 6 s or more and were not identified in Mascot with at least two spectra per feature and mass error lower than 5 ppm. The variations in retention time were compensated by expanding the retention time window for each precursor by 1 min in the inclusion list.

The database searches were performed in Proteome Discoverer (v1.3.0.339; Thermo Scientific). Mascot algorithm (Matrix Science, London, UK) was used for the construction of inclusion lists for the combined peptide sample, whereas in the final collective search of all 16 analyzed samples, both Mascot [18] and Sequest [19] were used. The spectra were searched against UniProtKB/Swiss-Prot mouse database (16,686 sequences, accessed 15th of February 2013), appended with protein contaminants from cRAP (the common Repository of Adventitious Proteins, accessed 3rd of March 2011). The data was searched for tryptic peptides with Percolator decoy search mode, allowing maximum two missed cleavage sites, 5 ppm precursor mass tolerance, 0.5 Da fragment mass tolerance and 1% FDR. Finally, those proteins fulfilling the inclusion criteria of having at least two peptide spectral matches and at least one unique identified peptide were exported to Progenesis for quantification.

Spectral data from the 16 mass spectrometry runs were imported to Progenesis 4.0 for feature detection and data analysis. All LC–MS maps were aligned to the second injection of the combined sample and the feature detection was performed by automatic peak picking algorithm in default sensitivity mode. In peak picking, the maximum charge of precursor was restricted to 3+ and the retention time window to 12 s. The linear section of the gradient was used in the analysis and the threshold for accepted normalization was 0.5–1.75 within all peptide features. The contaminants and features that were identified by less than two hits or had precursor mass tolerance higher than

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