Dietary Amino Acid Source Elicits Sex-Specific Metabolic Response to Diet-Induced NAFLD in Mice

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RESEARCH ARTICLE

Molecular Nutrition & Food Research
www.mnf-journal.com

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Scope: Non-alcoholic fatty liver disease (NAFLD) is a sexually dimorphic disease influenced by dietary factors. Here, the metabolic and hepatic effects of dietary amino acid (AA) source is assessed in Western diet (WD)-induced NAFLD in male and female mice.

Methods and results: The AA source is either casein or a free AA mixture mimicking the composition of casein. As expected, males fed a casein-based WD display glucose intolerance, fasting hyperglycemia, and insulin-resistance and develop NAFLD associated with changes in hepatic gene expression and microbiota dysbiosis. In contrast, males fed the AA-based WD show no steatosis, a similar gene expression profile as males fed a control diet, and a distinct microbiota composition compared to males fed a casein-based WD. Females are protected against WD-induced liver damage, hepatic gene expression, and gut microbiota changes regardless of the AA source.

Conclusions: Free dietary AA intake prevents the unhealthy metabolic outcomes of a WD preferentially in male mice.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most prevalent chronic liver disease, with a global prevalence of 25%,[1] and is recognized as a major public health issue. NAFLD is considered to be the hepatic manifestation of metabolic syndrome and strongly associated with obesity and type 2 diabetes. NAFLD includes a spectrum of liver diseases, including steatosis, or fatty liver disease, which is characterized by hepatic lipid accumulation. Steatosis is a benign disorder but predisposes the patient to severe forms, such as inflammatory steatosis (non-alcoholic steatohepatitis [NASH]), which promotes liver cirrhosis and cancer which is currently the fastest rising cause of cancer-related death worldwide.[2] NAFLD displays sexual dimorphism[3] in both mice and humans.[4] Epidemiological studies have indicated that pre-menopausal women are protected against NAFLD, whereas the incidence of NAFLD reaches the same level as men in post-menopausal women. Sex-specific gene expression profiles were recently identified in human NASH[5] and mouse models of NAFLD.[4]

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The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/mnfr.202300491

DOI: 10.1002/mnfr.202300491

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The development and progression of NAFLD is largely attributed to an excess of energy intake, mostly in the form of carbohydrates and fat, which enter the liver and result in triglyceride accumulation. Mechanistically, fructose stimulates hepatic de novo lipogenesis, a central mechanism of hepatic lipid accumulation in NAFLD that accounts for 26% of increased fatty acids in the liver.[5] High intake of saturated fat and cholesterol leads to disturbances in glucose and lipid metabolism and induces obesity and insulin resistance, which are strongly associated with NAFLD. Increasing evidence suggests that dietary protein intake may also have an impact on whole-body and hepatic homeostasis according to the quantity,[6] the amino acid (AA) composition, and/or origin (animal or vegetal) of the protein. However, the effects of dietary proteins are not fully understood and appear to depend on the life period,[7,8] the length of the dietary intervention,[9,10] the health status of the individual,[11,12] and the carbohydrate proportion in the diet.[11,13,14] A recent experimental study in mice showed that the total protein content in a diet is the main driver of the metabolic effect.[15] Low levels of dietary proteins lead to reduced food intake and body weight in mice, partially through inhibition of hypothalamic mammalian target of rapamycin (mTOR) signaling.[16] The metabolic health potency of a low protein diet has also been attributed to the reduced consumption of essential amino acids (EAAs), especially branched chain amino acids (BCAAs; e.g., leucine, valine, isoleucine). Selective restriction of dietary BCAAs improves glucose homeostasis and promotes weight loss in both humans and mice.[6,17] Reduced dietary levels of BCCA induced the same metabolic benefits as that of a protein-restricted diet,[6,17,18] suggesting that the AA composition of the diet plays a key role in the regulation of metabolic homeostasis. Interestingly, dietary BCAAs have been shown to restore liver metabolic homeostasis in ovariectomized female mice through the alpha isoform of the estrogen receptor (ERα).[19] Dietary proteins can also impact the gut microbiota, which can reciprocally alter the AA distribution along the gastrointestinal tract, influencing AA, glutathione, and lipid metabolism in the host.[20] Resident bacteria of the gut metabolize dietary AAs, leading to the production of metabolites, such as short-chain fatty acids (SCFAs) and branched-chain fatty acids (BCFAs), which have been shown to modulate the host metabolism.[21] Accordingly, the replacement of casein by an EAA mixture prevented diet-induced obesity in mice associated with changes in the gut microbiota composition.[22] Moreover, Nychyk et al.[23] recently demonstrated an interaction between specific dietary proteins and gut microbiota in regulating mouse body weight. The impact of dietary proteins on metabolic homeostasis may also be different according to their digestibility.[24,25] It is well recognized that, in addition to their role in protein synthesis, AAs are also involved in whole-body metabolic homeostasis through the activation of AA-sensing signals.[26] This suggests that, by driving the AA availability and absorption kinetics, protein digestibility could also play a role in metabolic homeostasis.[26]

Thus, the impact of dietary proteins has been mainly investigated in obesity and diabetes, but to a lesser extent in NAFLD development and progression. Moreover, though the liver is a sexually dimorphic organ and expression of metabolic genes, including those involved in AA homeostasis, is sexually dimorphic, the sex-specific effects of dietary proteins have never been described. In this study, we evaluated the impact of dietary free AAs in a Western diet (WD)-induced mouse model of NAFLD in both males and females. The effects of substituting casein for a free AA mixture mimicking the casein AA composition on whole-body metabolic homeostasis, hepatic gene expression, and gut microbiota were characterized.

2. Experimental Section

2.1. Mice and Diets

In vivo studies were performed in compliance with the European guidelines for the use and care of laboratory animals and approved by an independent ethics committee under authorization number 17430-2018110611093660. All mice were housed at 21–23 °C on a 12-h light/12-h dark cycle and had free access to the standard rodent diet (safe A04 U8220G10R from SAFE Augy, France) and tap water. Six-week-old male and female C57BL/6J mice were purchased from Charles Rivers Laboratories (L'Arbresle, France), kept 1 week for acclimatization, and then randomly allocated to the different experimental groups. Mice (n = 12 per group and per sex) were fed a chow diet (CD; 70% carbohydrate, 4% fat, and 14% protein, Figure S1a, Supporting Information), or a Western diet (WD; 61% carbohydrate, 20% fat, and 14% protein, Figure S1a, Supporting Information) for 15 weeks. CD and WD contained 14% of protein which was either casein or a free amino acid mixture mimicking the amino acid composition of casein (SAAJ INRAE, Jouy, see also Table S1, Supporting Information). Amino acid composition was measured in all diets using UV ionic chromatography (Eurofins, Nutrition Animal France). Food and water intake and body weight were measured weekly.

2.2. Blood and Tissue Sampling

Before starting the diet and prior to sacrifice, blood was collected from the submandibular vein in lithium heparin-coated tubes (Sarstedt, Nümbrecht, Allemagne). Plasma was isolated by centrifugation (1500 × g, 15 min, 4 °C) and stored at −80 °C. Following euthanasia by cervical dislocation, tissue samples (liver, subcutaneous, and perigonadal white adipose tissues [WAT], cecum) were collected, weighed, dissected, and used for histological analysis or snap-frozen in liquid nitrogen and stored at −80 °C until use.

2.3. Oral Glucose Tolerance Test (OGTT) and Plasma Insulin Measurement

OGTTs were performed after 12 weeks of the diet. Mice were fasted for 6 h before receiving an oral glucose load (2 g kg^{-1} body weight). Blood glucose was measured at the tail vein using an Accu-Check Performa glucometer (Roche Diabetes Care France, Mylan, France) 30 min before and 0, 15, 30, 60, 90, and 120 min after the glucose load. Thirty minutes before and 15 min after glucose gavage, 20 µL of blood was sampled from the tip of the tail vein to measure the plasma insulin concentration by ELISA (Merck, Darmstadt, Allemagne).

2.4. Plasma Biochemical Analyses

Plasma samples were assessed for aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), free fatty acids, triglycerides, total cholesterol, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) cholesterol levels using a Cobas Mira Plus biochemical analyzer (Roche Diagnostics, Indianapolis, IN, USA) (ANEXPLO Facility, Toulouse, France). The plasma insulin concentration was measured using the rat/mouse insulin ELISA kit (Merck). Blood glucose was measured by an Accu-Chek Performa glucometer (Roche Diabetes Care France, Mylan, France).

Plasma samples for amino acid quantification were treated using the Kairos amino acid kit (Waters, 720006533). Samples were then analyzed by LC-MS/MS using a Xevo TQ-S Micro (Waters) spectrometer.

2.5. Liver Neutral Lipid Analysis

Hepatic lipids were extracted as described previously.[27] Briefly, tissue samples were homogenized in Lysing Matrix D tubes with 2:1 v/v methanol/5 mM EGTA (ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid). Lipids corresponding to an equivalent of 2 mg of tissue were extracted in chloroform/methanol/water (2.5:2.5:2, v/v/v) in the presence of following internal standards: glyceryl trinonadecanoate, stigmasterol, and cholesteryl heptadecanoate (Sigma-Aldrich, Saint-Quentin-Fallavier, France). Total lipids were suspended in 160 µL ethyl acetate, and the triglycerides, free cholesterol, and cholesterol esters were analyzed by FID gas-chromatography using a focus Thermo Electron system with a Zebron-1 Phenomenex fused-silica capillary column (5 m, 0.32 mm i.d., 0.50 µm film thickness; Phenomenex, England) as described previously.[28] The oven temperature was programmed to increase from 200 to 350 °C at a rate of 5 °C min^{-1}, and the carrier gas was hydrogen (0.5 bar). The injector and detector were set to 315 and 345 °C, respectively.

2.6. Histology

Paraformaldehyde-fixed, paraffin-embedded liver tissue sections (3 µm) were stained with hematoxylin and eosin (H&E) or anti-α-smooth muscle actin (αSMA) antibody for histopathological analysis. The stained liver sections were analyzed blindly for steatosis, inflammation, and fibrosis. The histological features were grouped with the steatosis score evaluated according to Akpolat et al.[29] or inflammation andαSMA score evaluated according to Kleiner et al.[30]

2.7. Gene Expression

Total cellular RNA was extracted from liver samples using TRIzol reagent (Invitrogen). RNA was quantified using a NanoDrop (Nanophotometer N60, Implen). Two micrograms of total RNA were reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) for real-time quantitative polymerase chain reaction (qPCR) analyses. Primers for SYBR Green assays are presented in Table S2, Supporting Information. Amplifications were performed on an ABI Prism 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). RT-qPCR data were normalized to the level of TATA-box binding protein (TBP) messenger RNA (mRNA) and analyzed by LinRegPCR (v2016.1) to derive mean efficiency (N0).[31,32]

Gene expression profiles were obtained for eight liver samples per group at the GeT-TRiX facility (GenoToul, Genopole Toulouse Midi-Pyrenees) using Agilent Sureprint G3 Mouse GE v2 microarrays (8 × 60 K, design 074809, Agilent Technologies) according to the manufacturer's instructions. For each sample, cyanine-3 (Cy3) labeled cRNA was prepared from 200 ng of total RNA using the One-Color Quick Amp labeling kit (Agilent Technologies) according to the manufacturer's instructions, followed by Agencourt RNAClean XP (Agencourt Bioscience Corporation, Beverly, MA). Dye incorporation and cRNA yield were determined using a Dropsense 96 UV/VIS droplet reader (Trinean, Belgium). Next, 600 ng of Cy3-labeled cRNA were hybridized on the microarray slides following the manufacturer's instructions. Immediately after washing, slides were scanned on an Agilent G2505C Microarray Scanner using Agilent Scan Control A.8.5.1 software and the fluorescence signal extracted using Agilent Feature Extraction software (v10.10.1.1) with default parameters. Microarray data and experimental details are available in NCBI's Gene Expression Omnibus (GEO) database (accession number GSE (in progress)).

2.8. Bacterial DNA Extraction and Community Analysis by 16S rRNA Gene Sequencing

Cecal microbiota survey was performed as described by Chassaing et al.[33] DNA was extracted from frozen cecum using a QIAmp 96 PowerFecal QIAcube HTkit (Qiagen Laboratories) with mechanical disruption (Qiagen TissueLyser II). Briefly, 650 µL of prewarmed buffer PW1 was added to the samples. Subsequently, samples were thoroughly homogenized using bead-beating with a TissueLyser before centrifuging the plate at 4000 rpm for 5 min at 20 °C to pellet the particles. Four hundred microliters of supernatant were transferred to a new 96-well plate containing 150 µL of buffer C3. After mixing and incubating on ice for 5 min, centrifugation was performed at 4000 rpm for 5 min at 20 °C. Three hundred microliters of each supernatant was then transferred to a new 96-well S-block plate and 20 µL of Proteinase K added and incubated for 10 min at room temperature. The following steps were performed on a QIAcube high-throughput robot: addition of 500 µL of Buffer C4; DNA binding to a QIAmp 96 plate; column washing using 800 µL of AW1, 600 µL of AW2, and 400 µL of ethanol; and elution by adding 100 µL of ATE buffer.

Subsequently, 16S rRNA gene amplification and sequencing were performed using the Illumina MiSeq technology following the protocol of the Earth Microbiome Project (www.earthmicrobiome.org/emp-standardprotocols),[34] with some modifications. Briefly, the 16S rRNA genes, region V4, were PCR-amplified from each sample using a composite forward and reverse primer containing a unique 12-base barcode designed with the Golay error-correcting scheme used to tag PCR products from respective samples.[34] The forward primer 515F was used (5′-AATGATACGGCGACCACCGAGATCTACACGCT

XXXXXXXXXXXXTATGGTAATTGTGTGYCAGCMGCCGCGG TAA-3′); the italicized sequence was the 5′ Illumina adapter, the 12X sequence was the Golay barcode, and the bold sequence was the primer pad, the italicized bold sequence was the primer linker, and the underlined sequence was the conserved bacterial primer 515F. The 806R primer was 5′-CAAGCAGAAGACG GCATACGAGATAGTCAGCCAGCCGGACTACNVGGGTWTC TAAT-3′; the italicized sequence was the 3' reverse complement sequence of the Illumina adapter, the bold sequence was the primer pad, the italicized bold sequence was the primer linker, and the underlined sequence was the conserved bacterial primer 806R. PCR reactions consisted of 5PRIME HotMasterMix (Quantabio, Beverly, MA, USA), 0.2 µM of each primer, and 10–100 ng of template, and reaction conditions were set as follow: 3 min at 95 °C, followed by 30 cycles of 45 s at 95 °C, 60 s at 50 °C, and 90 s at 72 °C on a BioRad thermocycler. PCR products were then visualized by gel electrophoresis and quantified using Quanti-iT PicoGreen dsDNA assay (Thermo Fisher Scientific). A master DNA pool was generated from the purified products in equimolar ratios and subsequently purified with Ampure magnetic purification beads (Agencourt, Brea, CA, USA). The obtained purified pool was quantified using the Quanti-iT PicoGreen dsDNA assay, followed by sequencing using an Illumina MiSeq sequencer (pair-end reads, 2 × 250 bp) at the GENOM'IC core facility at Cochin Institut, Paris, France.

QIIME2-version 2022 was used to analyze 16s rRNA sequences.[35] These sequences were demultiplexed and quality filtered using the Dada2 method[36] with QIIME2 default parameters to detect and correct Illumina amplicon sequence data, generating a table of Qiime 2 artifacts. Next, a tree was generated using the align-to-tree-mafft-fasttree command for phylogenetic diversity analysis, and we computed alpha and beta diversity using the core-metrics-phylogenetic command. Principal coordinate analysis (PCoA) plots were used to assess variations between experimental groups (beta diversity). Alpha diversity was computed with the Evenness index. For the taxonomic analyses, features were assigned to operational taxonomic units (OTUs) with a 99% threshold of pairwise identity to the Greengenes reference database 13_8.[34–37] Unprocessed sequencing data are deposited in the European Nucleotide Archive under accession number PRJEB63317.

2.9. Proton Nuclear Magnetic Resonance (1H-NMR)-Based Metabolomics

Feces samples and liver polar extracts were prepared and analyzed using 1H-NMR-based metabolomics as described previously.[38] All spectra were obtained on a Bruker DRX-600-Avance NMR spectrometer (Bruker) on the AXIOM metabolomics platform (MetaToul). Data were mean-centered and scaled using the unit variance scaling prior to analysis with orthogonal projection on latent structure-discriminant analysis (O-PLS-DA). The O-PLS models were evaluated for goodness of prediction (Q^2 *Y* value) using *12*-fold cross-validation. The parameters of the final models are indicated in the figure legends. To identify metabolites responsible for discriminating between the groups, the O-PLS-DA correlation coefficients (r^2) were calculated for each variable.

2.10. Quantification and Statistical Analysis

Statistical analyses were performed using GraphPad Prism for Windows (version 9.3.1; GraphPad Software). Results were presented as the mean ± SEM. Differential effects were analyzed by a two-way ANOVA followed by Tukey's multiple comparison test. Except for the body weight survey (one-way ANOVA followed by Sidak's multiple comparison test), for plasma amino acid concentrations (unpaired *t* test with Welch's correction) and inflammation and fibrosis scores (Kruskal–Wallis test followed by Dunn's multiple comparison test) ($n = 12$ mice per group).

The steatosis score was based on the percentage of hepatocytes containing fat in each liver slice, where Grade 0 = <5% of hepatocytes containing fat; grade 1 = 5% to 32% of hepatocytes; grade 2 = 33% to 65% of hepatocytes; grade 3 = up to 65% of hepatocytes. The degree of inflammation was appreciated by counting the inflammatory foci in 10 distinct areas at 200X for each liver slice (Grade 0–3). Values represent the mean of 10 fields/liver slice.

The αSMA score was bases on the Akpolat et al.[29] score; where were determined as 0 = no staining or <3%; 1 = positive for 3–33%; 2 = positive for 34–66%; and 3 = positive for >66%.

Microarray data were analyzed using R (R Core Team, 2018) and Bioconductor packages[39] as described in GEO accession number GSE (in progress). Raw data (median signal intensity) were filtered, log2 transformed, and normalized using the quantile method.[40] A model was fitted using the limma lmFit function.[41] Pair-wise comparisons between biological conditions were applied using specific contrasts. A correction for multiple testing was applied using the Benjamini-Hochberg procedure[42] to control the false discovery rate (FDR). Probes with a fold change ≥2 or 1.5 and FDR ≤0.05 were considered to be differentially expressed between conditions. Hierarchical clustering was applied to the samples and the differentially expressed probes using 1-Pearson correlation coefficient as the distance and Ward's criterion for agglomeration. The clustering results are illustrated as a heatmap of expression signals. Gene Ontology analysis was performed using Metascape.[43]

The 1H-NMR-based metabolomics data were analyzed by considering correlation coefficients above the threshold defined by Pearson's critical correlation coefficient ($p < 0.05$; $|r| > 0.55$; for $n = 12$ per group) as significant. For illustration purposes, the area under the curve of several signals of interest was integrated and significance tested by 2-way ANOVA. For metabolite identification, 1H-13C heteronuclear single quantum coherence (HSQC) spectra were obtained for one representative sample for each biological matrix.

3. Results

3.1. Dietary Free Amino Acids in the WD Prevent Body Weight Gain in Both Males and Females

In a preliminary study, we unexpectedly observed that the WD in which casein (CAS) was substituted with a mixture of free AAs mimicking the casein AA composition did not induce body weight gain nor white adipose tissues (WAT) weight in male mice after 15 weeks (Figure S1b, Supporting Information). To confirm this significant impact of the dietary AA source on the metabolic

response to an obesogenic diet, an independent cohort of male and female mice was fed either a control diet (CD) or a WD in which the protein intake was either from CAS or the same free AA mixture designed upon the AA profile of casein over a 15-week period (**Figure 1a** and Figure S1a, Supporting Information). Data presented in Table S1, Supporting Information show that the four diets (CAS- or AA mixture-based WD or CD) did not relevantly differ in their AA composition. Food intake assessed weekly was similar between CAS-based WD and free AA mixture-based WD-fed males and females (Figure S1c and S1d, Supporting Information). The plasma AA profiles of animals fed CAS- and AA mixture-based diets did not show drastic differences in males (Figures 1b and S1e, Supporting Information) or females (Figures 1c and S1f, Supporting Information) except for the level of glycine and glutamine, which were higher in both sexes and in females, respectively, only with the AA mixture-based WD (Figure 1b,c).

As expected, in both males and females, CAS-based WD feeding led to significant increases in body weight (Figure 1d,h) and subcutaneous and epididymal adipose tissue mass (Figure S2a and S2b, Supporting Information) compared to animals fed a CAS-based CD. The CAS-based WD also led to significantly decreased glucose tolerance (Figure 1e,i) and increased fasting glycemia (Figure 1f,j) and HOMA-IR (Homeostasis Model Assessment of Insulin Resistance) (Figure 1g,k) in both male and female mice. In contrast, and confirming our previous observations, the body and adipose tissue weights of males fed an AA mixture-based WD over a 15-week period did not increase compared to CD-fed males (Figure 1d and Figure S2a, Supporting Information). Plasma biochemical analyses revealed that the elevated levels of LDL-, HDL-, and total cholesterol observed in males fed a CAS-based WD compared to animals fed a CAS-based CD were significantly reduced in animals fed an AA mixture-based WD (Figure S2c, Supporting Information). In line with the improvement in body weight, male mice fed an AA mixture-based WD presented with significant amelioration of glucose tolerance (Figure 1e) reduced fasting glycemia (Figure 1f) and insulin-resistance index (Figure 1g) compared to males fed a CAS-based WD. In females, the substitution of casein by the AA mixture in the WD also significantly prevented the increase in body weight gain and subcutaneous WAT mass induced by 15 weeks of the WD (Figure 1h and Figure S2b, Supporting Information) (8.04 ± 0.57 vs. 5.94 ± 0.051 g of body weight gain). However, in contrast to males, plasma cholesterol (Figure S2d, Supporting Information) and glucose homeostasis (Figure 1i–k) were not improved by the AA mixture-based WD.

Taken together, these results suggest that replacement of casein by a free AA mixture prevents WD-induced body weight gain in both males and females, which is associated with improved glucose homeostasis and plasma lipid profiles in male mice.

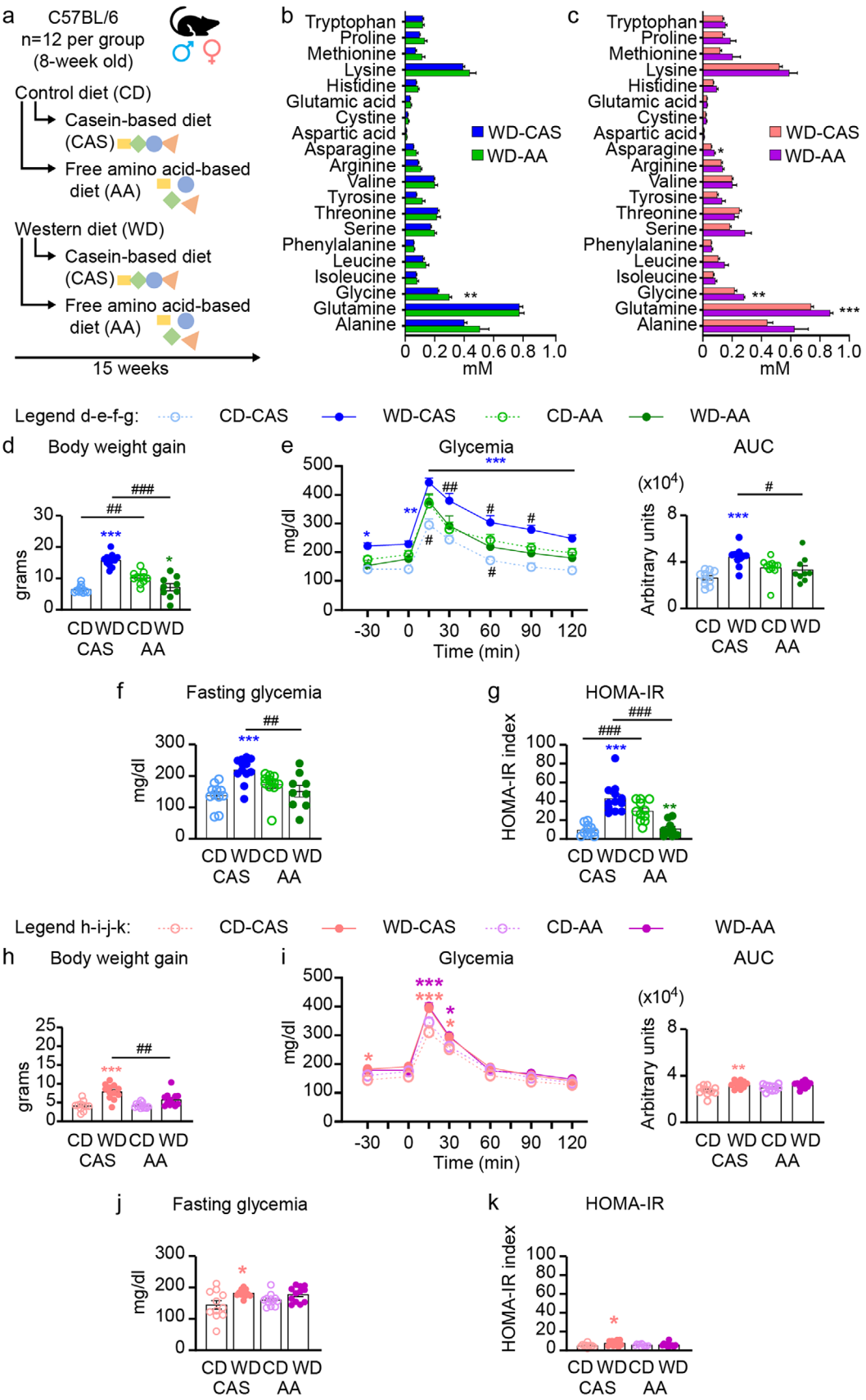
3.2. Dietary Free Amino Acids in the WD Prevent Hepatic Damage in a Sex-Specific Manner

Male mice fed a CAS-based WD for 15 weeks had increased liver weight compared to males fed a CAS-based CD (47%, Figure S3a, Supporting Information), indicating hepatic damage. As expected, histological analyses revealed hepatocellular vacuolization characteristic of steatosis in the livers of males fed a CAS-based WD (**Figure 2a**). The mean steatosis score was 2.3 $\pm$ 0.256 in males fed a CAS-based WD diet but insignificant in males fed a CAS-based CD and was confirmed by quantification of hepatic triglycerides (341.094 µg mg^{-1} $\pm$ 21.291 in males fed a CAS-based WD vs. 14.923 µg mg^{-1} $\pm$ 2.4 in males fed a CAS-based CD) and serum alanine aminotransferase (7-fold increase, Figure 2a). Free and esterified cholesterol levels were also increased in response to the CAS-based WD (Figure S3b, Supporting Information). In addition, RT qPCR analysis revealed increased expression of hepatic genes involved in lipogenesis (*Scd1, Fasn, Srebp1c*) and β-oxidation (*Cpt2, Acox1*) (Figure S3e, Supporting Information). Further analysis of liver inflammation by counting lobular inflammatory foci did not show any differences between livers from males fed a CAS-based CD or WD (Figure 2c). However, there was an increased expression of genes involved in hepatic inflammation, such as *Tnfα* and *Cxcl10*, and a trend in increased expression of *Il1β, F4/80*, and *Ccl2* in males fed a CAS-based WD compared to their controls suggesting an inflammatory response to WD (Figure 2d).

Next, we assessed stellate cell activation. Alpha smooth muscle actin cell staining (α−SMA) was slightly but significantly increased in the livers of males fed a CAS-based WD (Figure 2e) and correlated with the increased hepatic expression of collagens *Col1a1, Col3a1, Col4a1*, and *Tgf β* and *Mmp13* (Figure 2f). In contrast, most WD-induced hepatic lesions were reduced in males fed the AA mixture-based WD. Accordingly, males fed an AA mixture-based WD or CD had comparable levels of plasma markers of liver injury, steatosis scores, and hepatic triglyceride content (Figure 2a). Moreover, the elevation of free and esterified cholesterol observed in males in response to the CAS-based WD was abolished in males fed an AA mixture-based WD (Figure S3b, Supporting Information). As shown in Figure S3e, Supporting Information, the expression of genes involved in β-oxidation was not up-regulated in livers from males fed an AA mixture-based WD, whereas those involved in lipogenesis were less (*Scd1* and *Fasn*) or not (*Srebp1c*) induced compared to males fed a CAS-based WD. In addition, the hepatic expression of genes involved in inflammation and fibrosis was not increased in response to a WD in which casein was replaced by the free AA mixture (Figure 2d,f).

Compared with males, female liver damages in response to WD were slighter and did not change regardless of the AA source (Figures 2b and S3c-f, Supporting Information). The liver weight (Figure S3c, Supporting Information), steatosis score, and hepatic triglyceride content were only slightly increased in response to the WD and were similar between CAS-based and AA mixture-based WD (Figure 2b). Increases in hepatic levels of free and esterified cholesterol were also observed in females fed a CAS- or AA mixture-based WD (Figure S3d, Supporting Information). In line with the observed liver phenotype, the casein and AA mixture-based WD led to slight changes in the hepatic expression of genes involved in lipogenesis, β-oxidation, inflammation, and fibrosis (Figures S3f, S3g, and S3h, Supporting Information).

Next, we used ^{1}H-NMR-based metabolomics to characterize the hepatic metabolic profiles. We observed a significant discrimination between the hepatic metabolite patterns of males fed a CAS-based WD and males fed a CAS-based CD (Figure 2g, Table S3, Supporting Information). Coefficient plots derived from the



O-PLS-DA models revealed that the main discriminant metabolites were biliary acids, hypotaurine, glycero-phosphocholine, and succinate, which were decreased, whereas phosphocholine was increased in CAS-based WD-fed animals (Figure 2h and S4a, Supporting Information). In contrast, there were no notable differences in the metabolic profiles of males fed an AA mixture-based WD compared to males fed an AA mixture-based CD (Figure 2g and Table S3, Supporting Information). This effect on the hepatic metabolome was sexually dimorphic, as the O-PLS statistical models did not significantly discriminate the profiles of females fed a CD or WD regardless of the AA source (Figure S4b and Tables S3 and S4, Supporting Information).

Taken together, these results reveal that the replacement of casein with an equivalent mixture of free AAs is sufficient to prevent WD-induced NAFLD in male mice.

3.3. Dietary Free Amino Acids in the WD Change the Liver Gene Expression Pattern Mostly In Males

To further investigate the mechanisms involved in the sexually dimorphic prevention of WD-induced NAFLD associated with the AA change, we analyzed hepatic gene expression using microarrays. Principal component analysis (PCA) in males revealed differences in liver gene expression between males fed a CAS-based WD and males fed an AA mixture-based WD, whereas males fed either a CAS or an AA mixture-based CD exhibited fairly similar gene profiles (**Figure 3a**). In males, the CAS-based WD led to upregulation of 317 differentially expressed genes (DEGs; $p < 0.05$ and fold change >2) and the down-regulation of 137 genes (Figure 3b). In contrast, and as shown in Figure 3b, males fed an AA mixture-based WD had 57 up- and 5 down-regulated genes, providing molecular evidence of the metabolically ameliorating impact of the substitution of casein by the AA mixture in the WD. Hierarchical clustering of DEGs ($p < 0.05$ and fold change >2, 505 genes) highlighted three clusters with distinct hepatic gene expression between males fed a casein and AA mixture-based WD (Figure 3c). Genes from cluster 2 were up-regulated in response to WD regardless of the AA source (Figure 3d). Gene Ontology analysis revealed that these genes were mainly involved in lipid biosynthesis and peroxisome proliferator activated receptor (PPAR) signaling pathways (Figure 3e). Conversely, clusters 1 and 3 highlighted genes with down- and up-regulated expression, respectively, specifically in males fed a CAS-based WD, but not in males fed an AA mixture-based WD (Figure 3d). Cluster 3 comprised 358 genes mainly involved in cholesterol metabolism (4 DEGs/17), inflammation (35 DEGs/43), oxidative stress (18 DEGs/25), and extracellular matrix organization (12 DEGs/14), which correlates with the previously observed liver phenotype (Figure 3e). Interestingly, genes related to androgen catabolism and cellular response to AA stimulus were also significantly and specifically up-regulated in males fed a CAS-based WD compared to males fed a CAS-based CD. The expression of the representative genes in this cluster are presented in Figure S5a–d, Supporting Information. Cluster 1 included 173 down-regulated genes in response to the CAS-based WD but not the AA-based WD. These genes were mainly associated with complement activation (4 DEGs/6), response to steroid hormones (6 DEGs/11), and protein transport (14 DEGs/25) (Figure 3e and Figure S5e–g, Supporting Information). Figure S5h (Supporting Information) recapitulates the top 20 DEGs in each cluster. Interestingly, many genes form clusters 1 and 3 highlighted activation of the AA sensor mTOR (Figure S5h, Supporting Information). In addition, as dietary AA have been shown to activate hepatic ERα, we checked its expression levels and found an increase in *Erα* expression in response to WD only with the AA-based diet (Figure 3k).

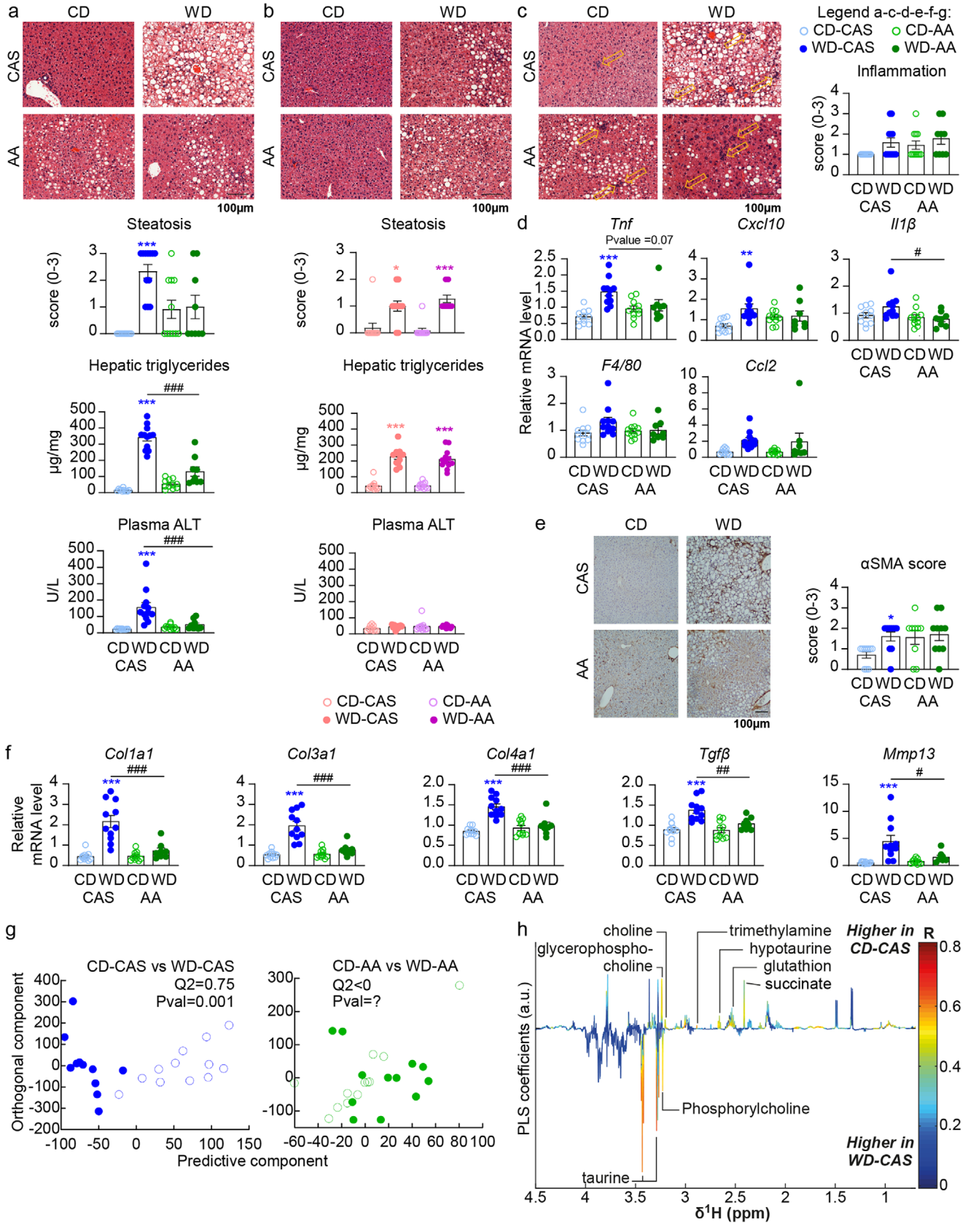
In contrast, in females, the AA source did not significantly affect the hepatic transcriptome. PCA could not discriminate the various animal groups (CAS-based WD or CD, AA mixture-based WD or CD; Figure 3f). However, we observed slight changes in hepatic gene expression in response to WD regardless of the AA source. The number of DEGs in response to the CAS-based WD were higher than in response to an AA-based WD (246 genes up- and 85 genes down-regulated with CAS-WD vs. CAS-CD and 102 genes up- and 32 down-regulated with AA-WD vs. AA-CD; Figure 3g). The heatmap resulting from the hierarchical clustering shown in Figure 3h highlighted two clusters of genes that were up- or down-regulated in a similar manner after 15 weeks of being fed a CAS- or AA mixture-based WD (Figure 3h,i). Pathway enrichment analysis (Figure 3j) revealed that down-regulated genes were mainly related to thyroid hormone metabolism and cholesterol and phospholipid biosynthesis. Up-regulated genes were involved in inflammation (27 DEGs/30) and extracellular matrix organization (10 DEGs/11). Figure S5j, Supporting Information, recapitulates the top 20 DEGs in each cluster.

Taken together, our results show that the replacement of casein by an AA mixture in a WD results in male-specific alleviation of WD-induced changes in hepatic gene expression.

3.4. Dietary Free Amino Acids in the WD Disturb the Intestinal Microbiota Mainly in Males

In both males and females, the cecum weights of animals fed a CAS-based WD were lower than the cecum weights of animals fed a CAS-based CD, but no change was observed between the cecum weights of animals fed an AA mixture-based WD and animals fed an AA mixture-based CD (Figure S6a and S6b, Supporting Information). Thus, we hypothesized that the source of

Figure 1. Dietary free amino acids in the WD prevent body weight gain in both males and females. a) Eight-week-old male and female C57BL6J mice were fed a control diet (CD) or a Western diet (WD) containing casein (CAS) or a free amino acid mixture (AA) mimicking the amino acid composition of casein (n = 12/group). b,c) Plasma amino acid concentration in males (b) and females (c) in response to 15 weeks of each diet. d) Relative body weight gain in males after 15 weeks of the diet. e–g) Oral glucose tolerance test (OGTT) and area under the curve (AUC) representing OGTT results (e), fasting glycemia (f), and HOMAR-IR (g) in males after 12 weeks of the diet. h) Relative body weight gain in females after 15 weeks of the diet. i,k) OGTT and AUC representing OGTT results (i), fasting glycemia (j), and HOMAR-IR (k) in females after 12 weeks of the diet. Results are presented as the mean ± SEM. *CD vs. WD and #CAS vs. AA; $*/^{\#}p < 0.05$, $**/^{\#\#}p < 0.01$, and $***/^{\#\#\#}p < 0.001$. Differential effects were analyzed by two-way ANOVA followed by Tukey's multiple comparison test, except for body weight survey (one-way ANOVA followed by Sidak's multiple comparison test) and plasma amino acid concentrations (unpaired *t* test with Welch's correction).



dietary AAs impacted the WD-induced gut microbiota disturbance and sequenced the cecum microbiota composition using Illumina-based rRNA 16S sequencing. Considering alpha diversity metrics, the bacterial richness and Shannon index increased in response to a WD in males fed a CAS-based diet (**Figure 4a**). When casein was replaced by the AA mixture, the species richness was slightly reduced, whereas the Shannon index was significantly lower in response to the WD compared to the AA mixture-based CD. Notably, free AA intake in animals fed a chow diet led to an increased Shannon index compared to casein intake, but it did not change the cecal microbiota richness. Bray Curtis distance analysis revealed a clear interaction between diet and AA source (Figure 4b). Fifty-eight percent of the microbiota differences are explained by the diet-AA source interaction (axis 1), whereas the diet alone (axis 2) and dietary AA source alone (axis 3) accounted for only 9% and 5% of the microbiota differences between groups, respectively. At the phylum level, males fed a CAS-based WD had a slight but significant decrease in abundance of Firmicutes and an increased abundance of Proteobacteria and Deferribacteres compared to males fed a CAS-based CD (Figure 4c and Figure S6c, Supporting Information). In contrast, in males fed an AA mixture-based WD, we observed an increased relative abundance of bacteria from the phylum Firmicutes and decreased abundance of Proteobacteria and Verrucomicrobia compared to males fed an AA mixture-based CD (Figure 4d and Figure S6c, Supporting Information). The main differentially abundant OTUs between males fed a CAS-based WD and males fed a CAS-based CD were affiliated with the *Bifidobacterium* and *Allobaculum* genera, the relative abundance of which was decreased. Inversely, the proportion of these genera were increased in response to the AA mixture based-WD (Figure 4d). AA source also led to variations in the differentially abundant OTUs in males fed a CD (Figure 4c,d). In females, though richness was similar in every condition, the response to the CAS-based WD was characterized by an increased Shannon index to a similar proportion as the CD considering the replacement of casein by the AA mixture (Figure 4e). Compared to males, the diet component had a stronger impact on the microbiota than the AA source component, as indicated by the contribution of this factor on the first axis (Figure 4f). At the phylum level, the CAS-based WD increased the level of Proteobacteria and decreased the Firmicutes proportion compared to the CAS-based CD. In females fed an AA mixture-based WD, there were no differences at the phylum level compared to females fed an AA mixture-based CD (Figure 4g and Figure S6d, Supporting Information).

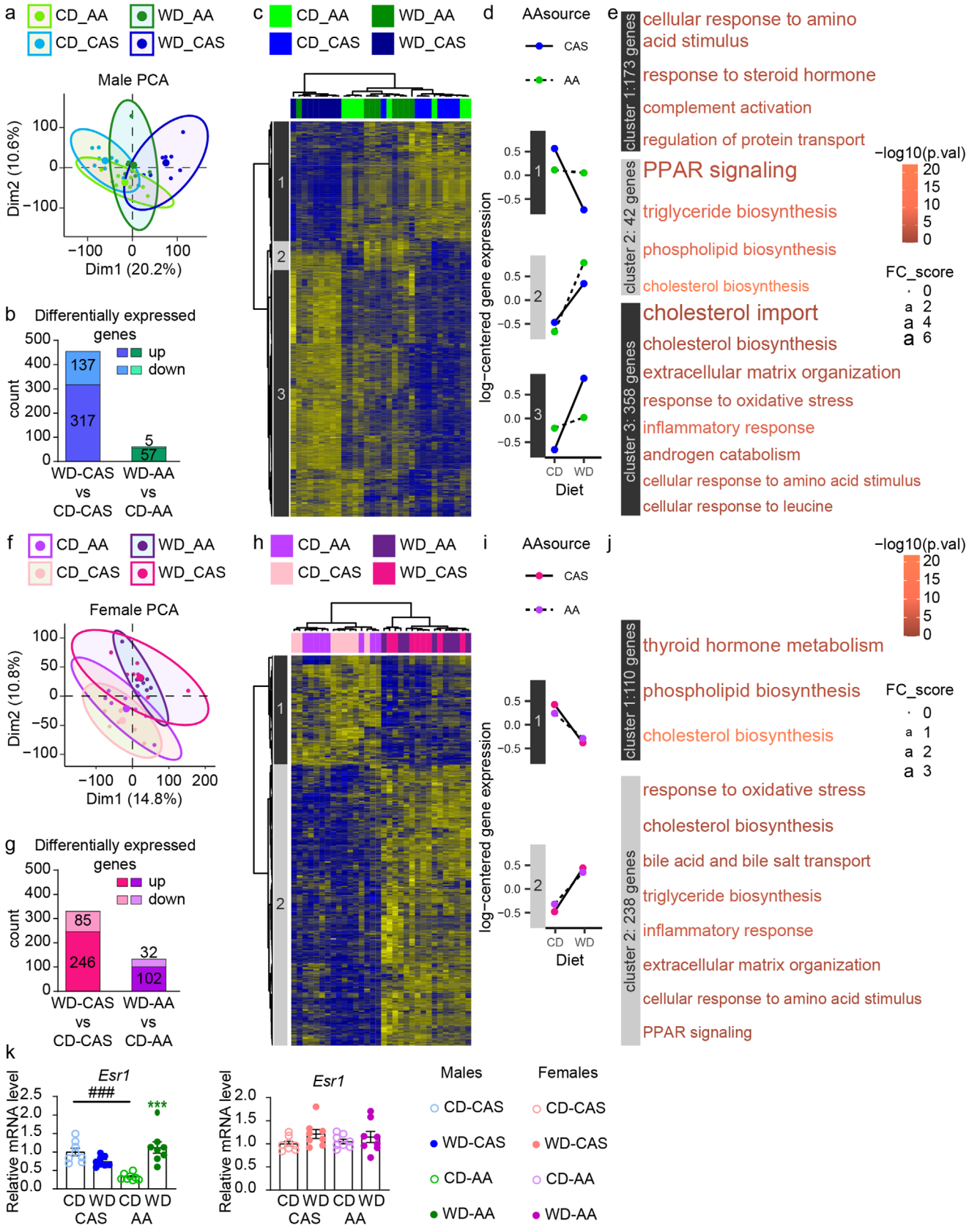
Fecal metabolic profiles were obtained in males to assess the metabolic consequences of the observed changes in the composition of the gut microbiota. PCA of the whole ^{1}H-NMR based metabolic profiles showed a clear separation between AA- and CAS-fed males on the 1st principal component, illustrating that the AA source strongly affected microbiota metabolism in both CD- and WD-fed animals. In addition, the 2nd principal component showed a distinct clustering of CD- vs. WD-fed animals, meaning a significant effect of the diet component (WD- vs. CD; Figure 4h). Discriminating O-PLS-DA models confirmed significant differences between all experimental groups (Figure 4i and Table S4, Supporting Information). In particular, a much higher fecal level of an unknown metabolite was present in CAS-fed males compared to AA-fed males, independent of the diet component (Figure S6e, Supporting Information). WD-fed mice had lower fecal glucose levels than CD-fed mice, independent of the dietary AA source (Figure S6e, Supporting Information). WD-fed male mice also had higher fecal bile acid concentrations than CD-fed male mice and, interestingly, bile acid perturbations were stronger in CAS-fed males compared to AA-fed males (Figure S6e, Supporting Information). Similarly, succinate levels were decreased only in WD-CAS vs. CD-CAS animals, whereas no difference was observed in AA-fed males (Figure S6e, Supporting Information). Overall, this result suggests that both the dietary AA source and dietary component affect the intestinal microbiota metabolism, with a potential interaction of both factors on fecal succinate and bile acid levels.

4. Discussion

Recent work suggested that dietary proteins contribute to the development of metabolic disease according to their quantity, AA composition, origin, and/or digestibility, which drives the AA availability.[9–11,13–15,24,44,45] However, little is known about the effects of dietary proteins on liver metabolism. Here, we assessed the metabolic and hepatic effects of the dietary AA source in a WD-induced mouse model of NAFLD. We postulated differential AA availability between dietary free AAs and casein that may impact metabolic homeostasis and associated hepatic gene expression in response to a WD. As NAFLD is a sexually dimorphic disease and hepatic AA homeostasis is also sexually dimorphic,[3,4,19] we studied both male and female mice.

In addition to the previously described effects of protein quantity, origin, and AA composition, we found that the metabolic outcomes of a WD are further influenced by the dietary AA source

Figure 2. Dietary free amino acids in the WD prevent hepatic damage in a sex-dependent manner. a,b) Representative histological sections (magnification ×100) of liver stained with hematoxylin and eosin (H&E), estimated liver steatosis score, hepatic triglyceride content, and plasma alanine aminotransferase (ALT) levels of males (a) and females (b) in each group (n = 12 per group). c) Inflammatory score for histological liver sections. Each tissue section was analyzed for 10 microscopic fields (magnification x200) to determine the mean number of inflammation foci per field (n = 12 per group). d) Hepatic mRNA expression of inflammatory genes measured by RT-qPCR in males from each group (n = 12/group). e) Representative histological sections (magnification ×100) of liver stained with αSMA and the estimated αSMA score for males in each group (n = 12/group). f) Hepatic mRNA expression of fibrotic genes measured by RT-qPCR in males from each group (n = 12/group). g) Orthogonal projection on latent structure-discriminant analysis (O-PLS-DA) score plots derived from the 1H-NMR spectra of aqueous hepatic extracts from males fed a casein-based WD vs. casein-based CD (g, left panel) and from males fed an AA mixture-based WD vs. AA mixture-based CD for 15 weeks (g, right panel). Q2 represents the goodness of fit for the PLS-DA models, and p-values were derived using 1000 permutations of the Y matrix. h) Coefficient plots derived from the O-PLS-DA model in males fed casein-based diets. Metabolites are color-coded according to their correlation coefficient, with red indicating a very strong positive correlation (R^2 > 0.65). Data are presented as the mean ± SEM. * CD vs. WD and # CAS vs. AA; $*/^{\#} p < 0.05$, $**/^{\#\#} p < 0.01$, and $***/^{\#\#\#} p < 0.001$. Differential effects were analyzed by two-way ANOVA followed by Tukey's multiple comparison test, except for inflammation and fibrosis scores, which were analyzed by a Kruskal–Wallis test followed by Dunn's multiple comparison test.



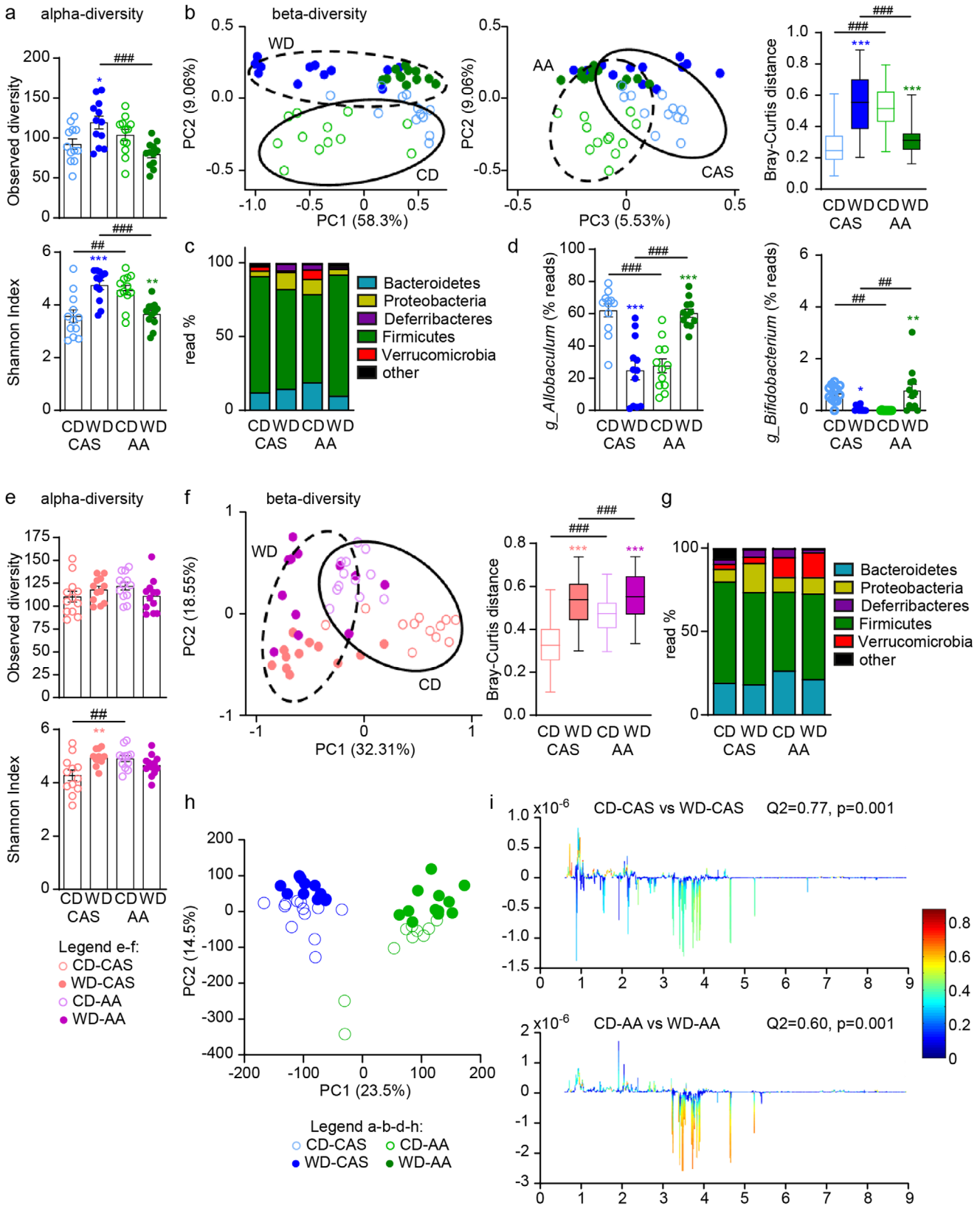
in a sexually dimorphic manner. Previous experimental studies have assessed the impact of an AA mixture-based diet on the metabolic disturbances induced by a high fat diet (HFD) in rodents.[46] However, in their experimental design, the authors did not compare their results with diets based on casein. Altering the source of dietary proteins has also been shown to have a major impact on the metabolic effects of obesogenic diets. Notably, a mixture of diversified proteins exacerbates obesity and insulin resistance induced by a high-fat high-sucrose diet, primarily through changes in gut microbiota composition and activity.[47]

In the present study, we found that the body weight gain, glucose intolerance, fasting hyperglycemia, and insulin-resistance observed in males fed a casein-based WD over a 15-week period did not occur when dietary casein was substituted with a free AA mixture mimicking the AA composition of casein. Similar results were obtained in females, though their metabolic response to a casein-WD was lower than that observed in males. Next, we observed a sexually dimorphic response to the dietary AA source at the hepatic level. Compared to males fed a casein-based WD that developed NAFLD associated with specific metabolites and gene expression pattern, male mice fed an AA mixture-based WD had a healthy hepatic phenotype. In contrast replacing casein by the AA mixture in the WD did not change the hepatic transcriptome in females. Liver gene expression analysis showed that the up-regulation of genes observed in males fed a casein-based WD and involved in inflammation (*Anaxa2, Lgals1, Lcn2*), lipogenesis (*Scd1, Fasn*, *Srebp1c, serpin, lipin*), fatty acid metabolism (*Cd36, Aldh3a2, Cpt2, Acox1*), oxidative stress (*Gpx4, Nqo1*), and fibrosis (*Eph, Mmp2, col1a1*) did not occur in the livers of males fed an AA mixture-based WD. In males fed an AA-based WD, metabolomic profiling revealed an increase in hepatic glycine and glycero-phosphocholine levels that were not observed in casein-based WD fed males. Low levels of plasma glycero-phosphocholine have been reported in NAFLD and NASH patients.[48–50] Glycine is a non-essential AA that was recently reported to be a limiting substrate for the synthesis of anti-oxidant glutathione (GSH) in NAFLD subjects, and plasma glycine levels have been negatively correlated with hepatic steatosis in both mice and humans.[51] Comparing germ-free and conventionally housed mice, Mardinoglu et al.[20] suggested that the gut microbiota may use glycine to support its growth, which leads to a decrease in glycine levels and reduced GSH biosynthesis.

The present work shows that the ameliorating effect of the AA mixture in response to a WD was associated with changes in gut microbiota composition and activity. Various preclinical studies have reported an impact of WD on the diversity and composition of microbial species that shape the gut microbiota.[52,53] Here, in males, the casein-based WD led to a decreased cecum weight associated with increased alpha and beta diversity. The replacement of casein by the free AA mixture in the WD led to a decrease in alpha diversity indexes. The gut microbiota of males fed a casein-based WD and AA mixture-based WD differed by the relative abundance of Proteobacteria and Firmicutes at the phylum level, which were decreased and increased, respectively, in animals fed an AA mixture-based WD. The microbiota of males fed an AA mixture-based WD was similar to that of males fed a casein-based CD. These observations are consistent with the similar phenotype of these two animal groups. The switch to the free AA mixture also led to a significant change in gut microbiota composition and abundance in CD-fed males. Thus, it is possible that the gut microbiota of AA-based WD-fed mice alters the dietary nutrient supply reaching the host. To further support this hypothesis, Nychyk et al.[23] recently showed a complex dietary interplay between dietary protein type and fat in the gut that influences microbiota composition, leading to improved metabolic outcomes of a HFD.

Protein digestibility has been reported to influence energy and metabolic homeostasis because it conditions the AA availability that can reach the colon and be used by the gut microbiota as a source of carbon nitrogen and energy,[54–57] contributing to its composition, structure, and function.[58] AAs can be metabolized by the gut microbiota into various products, such as SCFAs, polyamines, hydrogen sulfate, phenols, and indoles, that negatively or positively impact the host health.[57] Therefore, we hypothesized that the replacement of casein with an AA mixture provided a substantial loss of AAs reaching the colon, which subsequently leads to different gut microbiota composition and microbial metabolites, resulting in a differential effect on host metabolism. Numerous studies have reported the role of gut microbiota in NAFLD and obesity through its metabolites acting on the liver physiology and the integrity of the intestinal barrier.[59–61] In line with the observed changes in the composition of the gut microbiota, we found changes in the fecal metabolome of male mice fed an AA-based diet compared to a casein-based diet. Compared to males fed a casein-based WD, males fed an AA-based diet had higher fecal levels of succinate and lower levels of biliary acids. The primary bile acids synthetized in the liver are converted into secondary bile acids by microbial modifications in the gut. The interaction between bile acids and the gut microbiota regulates numerous metabolic pathways in the host.[62] In humans, increased fecal bile acid levels have been reported in NASH patients,[63,64] whereas in obese NAFLD patients with liver fibrosis, fecal bile acid levels decreased as

Figure 3. Dietary free amino acids in the WD change the liver gene expression pattern only in males. a–f) data from a microarray experiment performed with liver samples from male (a–e) and females (f–i) mice ($n = 8$/group). a,f) Principal component analysis (PCA) score plots of the whole transcriptomic dataset in the livers of male (a) and female (f) mice ($n = 8$/group). Each dot represents an animal projected onto first (horizontal axis) and second (vertical axis) PCA variables. b,g) Numbers of differentially up- and down-regulated genes in males (b) and females (g) fed a casein-based WD vs. a casein-based CD or fed an AA mixture-based WD vs. an AA mixture-based CD. c,h) Heatmap and hierarchical clustering showing the definition of three gene clusters in males ($p \leq 0.05$ and fold change >2) and two gene clusters in females ($p \leq 0.05$ and fold change >1.5). d,i) Representation of the mean cluster profiles in males (d) and females (i). e,j) Gene Ontology enrichment of selected GO categories in each heatmap cluster from males (e) and females (j). The size of the font is related to the score based on a log base 2 numbers of genes enriched and the color gradients of characters represent the –log base 10 value of the probability of the test for p [X > x]. k) Liver expression of the alpha isoform of the estrogen receptor (Esr1) in male and female mice ($n =$ 8 animals per group). Data are presented as the mean ± SEM. * CD vs. WD and # CAS vs. AA; ***/### $p < 0.001$. Differential effects were analyzed by two-way ANOVA followed by Tukey's multiple comparison test.



fibrosis progresses.[65] Fecal succinate can be produced by microbial fermentation of carbohydrates and AAs. Succinate, as well as acetate, propionate, and butyrate, play a great role in inter-organ crosstalk by regulating gut integrity and improving liver and peripheral tissue function and metabolism.[66] Recent studies have reported that succinate improves glucose homeostasis in mice through the induction of intestinal neoglucogenesis[67] and thermogenesis.[68]

Dietary proteins are involved in energy and metabolic homeostasis through the utilization of AA carbon chains,[69] and mTOR is one of the main AA sensors.[70,71] The mTOR signaling pathway was shown to be involved in liver steatosis in mice with diet-induced obesity.[72] In our study, among the genes strongly up-regulated in males fed a casein-based WD and not in males fed an AA mixture-based WD, many are compatible with activation of the mTOR pathway, such as *Srebp1c, Serpin 1*, and *Cd36*, which are involved in lipid metabolism; *Col1a1*, which is involved in the fibrotic process; *Ephb2*, which correlates with NAFLD development and progression[73,74]; and *Anxa2* protein, which is known to induce fluctuations in autophagy.[75] However, one cannot exclude that the free AA mixture led to acute but transient hepatic mTOR activation that is not sufficient to up-regulate lipogenesis in males fed a WD.

Dietary AAs have also been shown to regulate the transcriptional activity of ERα in the liver through an mTOR-dependent mechanism.[19,76,77] This AA-dependent activation of ERα is required for the regulation of reproductive functions in response to nutrient availability and the control of lipid homeostasis in females.[19] In our study, the hepatic expression of *Er*α was increased in males fed the AA mixture-based WD. One could speculate that, in males fed an AA mixture-based WD, ERα is one of the players driving improvements in metabolic and hepatic homeostasis. The different kinetics of plasma AA release between the free AA-based and casein-based WD[44,78] suggests that free AAs are rapidly absorbed in the upper part of the digestive tract, leading to a drastic but transient increase in plasma AA levels reaching the liver, which results in ERα activation, rather than mTOR activation. Unfortunately, in our study blood samples were not collected in the postprandial period, and thus plasma analysis did not reveal changes in the AA plasma release between animals fed a casein and an AA mixture-based diet.

Another interesting finding in our study was the sexually dimorphic hepatic response to the AA mixture-based WD. In contrast to males, the hepatic phenotype and gene expression profile were not different between females fed a casein-based WD or an AA mixture-based WD. Though males are usually more sensitive to a WD than females,[4] the substitution of casein with the AA mixture appears to exert beneficial hepatic effects only in males. One cannot exclude that the differences observed between sexes are due to different time courses in the development of the disease.[4] In accordance with the female hepatic phenotype, the impact of the four diets on the gut microbiota was less obvious than in males, which suggests that the absence of microbiota dysbiosis may explain the female phenotype after the AA-based WD. Moreover, the female hepatic expression of *Er*α was not changed regardless of the AA source. ERα may be involved in the observed differential response between males and females.

Collectively, our data showed that free dietary AA intake prevents the unhealthy metabolic outcomes of a WD in a sex-specific manner that may involve the gut microbiota. Our study provides a new basis for the design of nutritional interventions that could limit the progression of NAFLD.

4.1. Limitations of the Study

Although our study was performed on 12 animals per group and in both sexes, it has some limitations.

First, we did not clearly identify mechanisms that drive the beneficial effects of free dietary AAs in male mice, an aspect that deserves further investigations. The question remains whether the free AA mixture exerts a direct hepatic effect through AA sensors or an indirect impact involving the gut microbiota. Second, one cannot exclude that only one or a few of the AAs present in the mixture are responsible for the observed beneficial effects. To confirm the role of AA availability in the ameliorating effect of the free AA source, further study comparing the effects of proteins with different digestion rates (casein vs. whey) on the response to a WD could be considered. Finally, it remains to be clarified whether our observations can be translated to an AA mixture mimicking the composition of proteins other than casein and to other obesogenic diets, such as HFD.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

The authors thank all members of the EZOP staff for their help with this project. The authors thank the GeT-Trix Genotoul facility and Anexplo facilities for their excellent work on plasma biochemistry. All MS and NMR experiments were performed using instruments of the Metatoul-AXIOM platform, partner of the national infrastructure of metabolomics

Figure 4. Dietary free amino acids in the WD disturb the intestinal microbiota, mainly in males. a–g) Cecal content microbiota analysis by 16S RNA sequencing. a,e) Alpha-diversity indexes in males (a) and females (e) from each group (n = 12/group). b,f) Beta-diversity in males (b) and females (f) using principal coordinates analysis (PCoA) based on the Bray-Curtis distance matrix of fecal microbiota. c,g) Relative abundances of the main phyla recovered in males (c) and females (g). d) Two representative genera identified as differentially abundant in males using ANCOM. n = 12 mice per group, *CD vs. WD and #CAS vs. AA; $*/^{\#}p < 0.05$, $**/^{\#\#}p < 0.01$, and $***/^{\#\#\#}p < 0.001$. h–i) Cecal metabolome analysis by H^1-NMR. h) Orthogonal projection on latent structure-discriminant analysis (O-PLS-DA) score plots derived from the 1H-NMR spectra of aqueous cecal extracts from males fed a casein-based WD vs. casein-based CD and from males fed an AA mixture-based WD vs. casein-based CD for 15 weeks. i) Coefficient plots derived from the O-PLS-DA model in males fed casein- or AA mixture-based diets. Metabolites are color-coded according to their correlation coefficient, with red indicating a very strong positive correlation ($R^2 > 0.65$). For alpha-diversity metrics and relative abundances of taxa, differences across groups were tested using ANOVA followed by Tukey's post-hoc test when $p < 0.05$. For beta-diversity, statistical differences were evaluated using PERMANOVA followed by a pairwise test.

and fluxomics: MetaboHUB [MetaboHUB-ANR-11-INBS-0010, 2011]. The authors also thank X. Blanc from the INRAE SAAJ–RAF team (Jouy-en-Josas, France) for his technical support with the pellet preparation. C.R. was supported by FRM (ENV202109013962). This work was funded by a grant from Région Occitanie (21019900 "LEARN").

Conflict of Interest

The authors declare no competing interests.

Author Contributions

L.G.P. and A.F. contributed equally to this work. C.R. designed experiments, performed experiments, analyzed the data, and wrote the manuscript. S.E.S, L.E., C.R., C.M., M.H., A.P., F.L., V.A.B., P.P., J.G, Y.P., and C.N. contributed to experiments and data analysis. S.E.S, T.L., F.S, P.F, B.C., H.G., and N.L. provided reagents, contributed to experiment design and supervision. A.F. and L.G.P. designed and supervised the project, analyzed the data, and wrote the manuscript. All authors revised the manuscript.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

amino acids, liver metabolism, microbiota, NAFLD, sex dimorphism

Received: July 13, 2023
Revised: September 21, 2023
Published online:

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