The calcium-sensing-receptor (CaSR) in adipocytes contributes to sex-differences in the susceptibility to high fat diet induced obesity and atherosclerosis

Svenja Adam,[a,b,m] Sanne L. Maas,[a,b,m] Rosanna Huchzermeier,[a,b] Leonida Rakateli,[a,b] Kathrin Abschlag,[a,b] Mathias Hohl,[c] Liangliang Liao,[d,e] Matthias Bartneck,[d,f,g] Margee Teunissen,[h] Kristiaan Wouters,[h] Donato Santovito,[i,j] Joachim Jankowski,[a,b] Erik A. L. Biessen,[a,b,k] and Emiel P. C. van der Vorst[a,b,i,l,*]

[a]Institute for Molecular Cardiovascular Research (IMCAR), RWTH Aachen University, Aachen 52074, Germany
[b]Aachen-Maastricht Institute for CardioRenal Disease (AMICARE), RWTH Aachen University, Aachen 52074, Germany
[c]Klinik für Innere Medizin III, Universität des Saarlandes, Homburg, Germany
[d]Department of Medicine III, University Hospital RWTH Aachen, Aachen, Germany
[e]Japan Union Hospital of Jilin University, Changchun, China
[f]DWI-Leibniz Institute for Interactive Materials, Aachen, Germany
[g]Institute of Technical and Macromolecular Chemistry, RWTH Aachen University, Aachen, Germany
[h]Department of Internal Medicine, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University Medical Centre, Maastricht 6229 ER, the Netherlands
[i]Institute for Cardiovascular Prevention (IPEK), Ludwig-Maximilians-University Munich, Munich 80336, Germany
[j]German Centre for Cardiovascular Research (DZHK), partner site Munich Heart Alliance, Munich 80336, Germany
[k]Department of Pathology, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University Medical Centre, Maastricht 6229 ER, the Netherlands
[l]Interdisciplinary Center for Clinical Research (IZKF), RWTH Aachen University, Aachen 52074, Germany

eBioMedicine
2024;107: 105293
Published Online xxx
https://doi.org/10.1016/j.ebiom.2024.105293

Summary

Background Female mice are more resistant to obesogenic effects of a high-fat diet (HFD), compared to male mice. Although the underlying mechanisms are poorly understood, sex hormones seem to play an important role. Interestingly, the activity of the oestrogen receptor-α (ERα) is affected by the calcium-sensing-receptor (CaSR). Therefore, we investigated sex-differences upon diet-induced obesity and the role of adipocyte-specific CaSR herein.

Methods Adipocyte-specific *Casr* deficient mice (*AdipoqCre*$^{+}$*Casr*^{flox}) and control mice (*Casr*^{flox}) were injected with AAV8-PCSK9 to make them prone to develop atherosclerosis and fed an obesity-inducing diet for 12 weeks.

Findings Female mice have lower visceral white adipose tissue (vWAT) mass compared to male mice, while this sex-difference is abolished upon adipocyte-specific *Casr* deficiency. Furthermore, while females showed elevated levels of inflammatory cytokines and CD3^{+}CD8^{+} T cell accumulation in vWAT, compared to males, adipocyte-specific *Casr* deficiency abrogated this sex-phenotype and demonstrated an inhibition of inflammatory signalling pathways. The expression of *Erα*, as well as associated genes involved in adipocyte differentiation, was increased in female mice in a mostly adipocyte-specific *Casr* dependent manner. Interestingly, circulating lipid levels were reduced in female compared to male mice, which correlated with decreased atherosclerotic plaque formation. These systemic effects were abrogated upon adipocyte-specific *Casr* deficiency.

Interpretation Our findings indicate that female mice show a more pronounced vWAT dysfunction compared to males upon obesity. This sex effect is abolished upon adipocyte-specific *Casr* deficiency. In contrast, females show diminished atherosclerotic plaque formation compared to males, an effect that was abrogated by adipocyte-specific *Casr* deficiency.

Funding This work was supported by a grant from the Interdisciplinary Center for Clinical Research within the faculty of Medicine at the RWTH Aachen University, by the Corona Foundation, by the Deutsche Forschungsgemeinschaft (DFG), the BMBF and Free State of Bavaria and the DZHK.

*Corresponding author. Pauwelsstrasse 17, Aachen 52074, Germany.
E-mail address: evandervorst@ukaachen.de (E.P.C. van der Vorst).
[m]Authors share first authorship.

Keywords: Sex-differences; Adipocytes; Obesity; Inflammation; Calcium-sensing receptor; Atherosclerosis

Research in context

Evidence before this study

Obesity is accompanied by excessive deposition of visceral white adipose tissue (vWAT), due to hypertrophy and hyperplasia. This excessive vWAT deposition leads to a chronic inflammation of the tissue and thereby the vWAT becomes dysfunctional. Interestingly, the burden of obesity, depicted in the global prevalence, is higher in women than in men. However, most pre-clinical research still omits sex-based analyses, resulting in a knowledge gap regarding the possible sex dependency of underlying mechanisms. With the help of animal models it has been shown that sex hormones influence adipocyte development, lipolysis, as well as the inflammatory tone in response to obesity. Especially oestrogen seems to play a key role, as an ovariectomy results in an increased weight gain, which was rescued by administrating oestrogen. Interestingly, the activity of the oestrogen receptor α (ERα) can be affected by extracellular Ca^{2+}, at least in part in a membrane-bound calcium-sensing receptor (CaSR) dependent manner. *In vitro* experiments elucidated the involvement of the CaSR in both adipocyte proliferation and differentiation, as activation of the CaSR in human adipocytes and adipocyte progenitor cell lines significantly enhanced the differentiation and adipogenesis. However, so far the role of CaSR in adipocytes is mainly investigated *in vitro*, while *in vivo* observations and thereby potential sex-differences are scarce.

Added value of this study

In the current study we addressed the effects of sex on obesity-induced vWAT inflammation, lipid accumulation, and atherosclerosis formation *in vivo*. It could be demonstrated that the vWAT and subcutaneous WAT (sWAT) of female mice is more inflamed compared to male mice in a model of HFD-induced obesity. This sex-difference is highly influenced by CaSR as adipocyte-specific *Casr* deficiency could abrogate the elevated inflammation of the vWAT in females. Thereby, this study shows a clear role of CaSR in obesity-related sex-differences. Mechanistically, it could be shown that these effects are related to *Erα* expression and oestrogen signalling. However, it should be noted that in contrast to these local WAT effects, adipocyte-specific *Casr* deficiency resulted in elevated circulating lipid levels and atherosclerosis development.

Implications of all the available evidence

By assessing the possible translational relevance of our results to humans, we found that the single nucleotide polymorphism (SNP) rs7648041 in the *CASR* locus was significantly associated with body mass index (BMI) in humans ($P < 3.4 \times 10^{-6}$), suggesting a role of this receptor in regulating adiposity also in humans.

Although the local vWAT effect would suggest that CaSR inhibition would be a promising target to treat vWAT dysfunction especially in females, the resulting systemic elevation in lipid levels and atherosclerosis formation warrant caution for such approaches. Future studies are needed to elucidate whether CaSR expression on other adipose tissue related cells (e.g., immune cells) also play a role in vWAT inflammation, perhaps without affecting the systemic lipid levels that fuel cardiometabolic diseases.

Introduction

Obesity is accompanied by excessive deposition of visceral white adipose tissue (vWAT), due to hypertrophy and hyperplasia. This excessive vWAT deposition leads to a chronic inflammation of the tissue and thereby the vWAT becomes dysfunctional.[1] Interestingly, the burden of obesity, depicted in the global prevalence, is higher in women than in men.[2] Additionally, besides this sex-specific discrepancy in obesity prevalence, also the implications of excess weight gain on health has been shown to vary between sexes.[3,4] For example, men with obesity have an increased risk for metabolic syndrome and cardiovascular disease (CVD), compared to (premenopausal) women with obesity.[5] This protective effect seems to be mainly hormone related, as men with obesity have a decreased risk for metabolic syndrome and CVD, compared to post-menopausal women.[5,6] Although it seems clear that sex-differences are of great importance, most pre-clinical research still omits sex-based analyses, resulting in a knowledge gap regarding the possible sex dependency of underlying mechanisms.[7]

The most common pre-clinical mouse model for obesity studies is a diet-induced obesity (DIO) model, in which mice are fed a high-fat diet (HFD). So far only few studies have investigated the sex-specific effects in such model. However, it could already be observed that female C57BL/6 mice are more resistant to the obesogenic effects of HFD feeding, which is characterized by a significant reduction in weight gain over time compared to male mice.[8] This observation is one of the reasons why female mice are often excluded from obesity studies. Recently, another study demonstrated that HFD feeding in female mice was linked to an enhanced reduction in respiratory quotient, which suggests that female mice have a greater ability to utilize fat

in the diet as an energy source, compared to male mice.[9] Although it could be shown that neurological signalling, at least partially, plays a role in these sex-differences, detailed underlying mechanisms remain rather elusive.[9]

With the help of animal models it has been shown that sex hormones influence adipocyte development, lipolysis, as well as the inflammatory tone in response to obesity.[10] Especially oestrogen seems to play a key role, as an ovariectomy results in an increased weight gain, which was rescued by administrating oestrogen.[11] Additionally, deletion of the oestrogen receptor α (ERα) in adipocytes resulted in an increased adiposity in mice,[12,13] further confirming an important role for oestrogen metabolism in obesity. Furthermore, the activity of the ERα can be affected by extracellular Ca^{2+}, at least in part in a membrane-bound calcium-sensing receptor (CaSR) dependent manner, in a breast cancer cell line.[14]

The CaSR is a dimeric family C G-protein-coupled receptor (GPCR), which is ubiquitously expressed throughout the body.[15] Physiologically, the major function of the CaSR is the regulation of the calcium-homeostasis, by sensing blood calcium levels in the parathyroid gland and normalizing them by regulating the parathyroid hormone (PTH) secretion and urinary calcium excretion.[16–18] Remarkably, the influence of the CaSR on PTH secretion varies between sexes, which was shown in a parathyroid gland-specific *Casr* deficient mouse model in which an increase of secreted PTH was observed in female mice, compared to male mice.[19] Two cell types, adipocytes as well as adipocyte progenitor cells, which play a crucial role in obesity, also express the CaSR, although its role in these cell types and in adipose tissue is still not fully understood.[20] *In vitro* experiments elucidated the involvement of the CaSR in both adipocyte proliferation and differentiation, as activation of the CaSR in human adipocytes and adipocyte progenitor cell lines significantly enhanced the differentiation and adipogenesis.[21,22] However, so far the role of CaSR in adipocytes is mainly investigated *in vitro*, while *in vivo* observations and thereby potential sex-differences are scarce.

Therefore, in the current study we addressed the effects of sex on obesity-induced vWAT inflammation, lipid accumulation, and atherosclerotic lesion formation *in vivo*. The contribution of CaSR in these sex effects was further elucidated utilizing an adipocyte-specific *Casr*-deficient mouse model.

Methods

Animals

To generate adipocyte-specific *Casr* deficient mice, $Casr^{flox/flox}$ mice ("$Casr^{flox}$" kindly provided by Dr. W. Chang from the University of California San Francisco, USA) were crossed with inducible adiponectin promoter driven CRE-ERT2 mice, C57Bl/6-Tg (Adipoq-icre/ERT2) 1Soff/J ("*AdipoqCre*", Jackson Laboratory, stock No. 025124). All mice were on a C57BL/6J background. The mice were injected *i.p.* with tamoxifen (50 mg/kg body weight, Sigma–Aldrich; Cat#T5648 in Miglyol, Caelo) for 5 consecutive days to induce the deletion of *Casr* in adipocytes. This mouse model has already been validated previously.[23] Subsequently, mice were injected *i.v.* with adeno-associated virus vector-8 (AAV8) encoding a gain-of-function mutant proprotein convertase subtilisin/kexin type 9 (AAV8-PCSK9, 2.5×10^{11} GC/ml, Vector Biosystems; Cat#NM_153565) to induce PCSK9 overexpression and thereby inducing hyperlipidaemia, promoting atherosclerosis.[24] One week after the AAV8-PCSK9 injection the mice received an obesity-inducing diet (DIO, Sniff, Cat#D12492) containing 34,6% crude fat (0.92% Calcium) for 12 weeks. After 12 weeks of DIO feeding, mice were anesthetized (10 mg/kg Xylazin and 100 mg/kg Ketamin in 0.9% NaCl), the blood was collected via puncture of the retro-orbital plexus, and the vasculature was perfused with PBS (Sigma–Aldrich Cat#P7059). Both sexes were used for all experiments. All animal experiments were approved by the local authorities (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, Germany, approval number 81-02.04.2019.A363) and complied with the German animal protection law. Furthermore, the study was conducted complying with the ARRIVE guidelines.

Tissue processing

Protein was isolated from vWAT (perigonadal) (25 mg), subcutaneous WAT (sWAT) (25 mg) and livers (50 mg) by homogenization of the tissue with metal beads at 50 Hz for 5 min in cell lysis buffer (M-PER™ Mammalian Protein Extraction Reagent (ThermoFisher Scientific; Cat#78501) containing protease inhibitors (Halt™ Protease Inhibitor Cocktail; ThermoFisher Scientific; Cat#78425) and phosphatase inhibitors (Halt™ Phosphatase Inhibitor Cocktail; ThermoFisher Scientific; Cat#78428) using the TissueLyser LT (Qiagen)). Protein concentration was measured with a NanoDrop One Microvolume UV–Vis Spectrophotometer (ThermoFisher Scientific).

For staining's, vWAT (perigonadal) was embedded in paraffin, and 10 mm cut sections were stained with Haematoxylin & Eosin (H&E). Digital images were taken at a magnification of 40× or 100× using a Leica DFC320 digital camera (Leica Microsystems, Wetzlar, Germany). Adipocyte cell surface and cell diameter were quantified with Image J, in a semi-automated fashion using automated cell border detection. Any adipocytes with visible lacerations to the membranes are "closed" manually prior to continuing with the automated area quantification.

Intraperitoneal glucose and insulin tolerance test

An intraperitoneal glucose tolerance test (GTT) and an intraperitoneal insulin tolerance test (ITT) were performed to determine blood glucose levels and insulin sensitivity after 10 or 11-weeks of DIO feeding,

respectively. The mice were starved for 6 hours, followed by an *i.p.* injection of glucose (2 mg/kg, Roth) or human normal insulin (0.02 U/kg, Lilly). Glucose levels were measured from blood (5 μL), collected from the tail vain, with disposable blood glucose stripes and a handheld glucometer (CONTOUR®NEXT at baseline, 15, 30, 60, 90 and 120 minutes after glucose or insulin administration.

ELISA

Inflammatory cytokine level (IL-6, CCL2, and TNF) were measured in EDTA-plasma samples as well as vWAT (perigonadal), sWAT and liver lysates using commercially available ELISA kits (ThermoFisher Scientific; Cat#88-7064-77, Cat#88-7391-77, Cat#88-7324-77) according to the manufacturer's protocol.

PTH and Oestrogen were measured in EDTA-plasma samples as well as vWAT (perigonadal) using a commercially available ELISA kit (Antibodies.com; Cat#A78690 and AFG Bioscience; Cat#EK730734, respectively) according to the manufacturer's protocol.

Calcium measurement

Calcium levels were measured in vWAT (perigonadal) using a commercially available calcium assay kit (Abcam; Cat#ab102505) according to the manufacturer's protocol.

Flow cytometry

vWAT (perigonadal) was digested with Liberase™ (1 mg/ml, Sigma–Aldrich; Cat#05401020001) and mechanical disruption followed by exposure to erythrolysis. Cell subsets in the vWAT were analysed using the following combination of surface markers: anti-CD45 (Invitrogen; Cat#47-0451-82), anti-F4/80 (BD Bioscience; Cat#565410), anti-CD11b (BD Bioscience; Cat#550993), anti-CD11c (eBioscience; Cat#25-0114-82), anti-CD301 (Bio Rad; Cat#MCA2392A647T), anti-CD3e (Invitrogen; Cat#45-0031-82), anti-CD4 (eBioscience; Cat#17-0041-82) and anti-CD8 (eBioscience; Cat#57-0081-82). Cell populations in the vWAT were gated as: total leukocytes ($CD45^+$), macrophages ($CD45^+CD11b^+F4/80^+$), M2-like macrophages ($CD45^+CD11b^+F4/80^+CD11c^-CD301^+$), CD3+ T cells ($CD45^+CD3^+$), $CD4^+$ T-cells ($CD45^+CD3^+CD4^+$), $CD8^+$ T-cells ($CD45^+CD3^+CD8^+$). Cell populations and marker expression were analysed using a FACSCanto™ II with FACSDiva software (BD Biosciences) and analysed with FlowJo (BD Bioscience).

qPCR

RNA was isolated from vWAT (perigonadal) and sWAT using the miRNeasy mini kit (Qiagen; Cat#217084), and from 3T3-L1 cells using the Quick-RNA Microprep kit (Zymogen; Cat#R1051), according to the manufacturer's protocol. The RNA was reverse-transcribed into cDNA using M-MLV RT (Invitrogen; Cat#M1705). qPCR was performed using PowerUp™ SYBR™ Green Master Mix (ThermoFisher Scientific; Cat#A25741) and real time specific primer pairs (2 μM/μL, Eurofins Genomics), using the following sequences: *Adipoq*: F: 5′-TGACGACACCAAAAGGGCTC-3′ and R: 5′-CACAAGTTCCCTTGGGTGGA-3'; *aP2*: F: 5′-TGAAATCACCGCAGACGACAGG-3′ and R: 5′-GCTTGTCACCATCTCGTTTTCTC-3'; *Atgl*: F: 5′-CAACGCCACTCACATCTACGG-3′ and R: 5′-GGACACCTCAATAATGTTGGCAC-3′; *β-actin*: F: 5′-CAACGAGCGGTTCCGATG-3′ and R: 5′-GCCACAGGATTCCATACCCAA-3′; *Cd36*: F: 5′-GCCAAGCTATTGCGACATGA-3′ and R: 5′-AAAAGAATCTCAATGTCCGAGACTTT-3'; *Cebpa*: F: 5′-ATAGACATCAGCGCCTACATCGA-3′ and R: 5′-GTCGGCTGTGCTGGAAGAG-3'; *Esr1*: F: 5′-AATTCTGACAATCGACGCCAG-3′ and R: 5′-GTGCTTCAACATTCTCCCTCCTC-3'; *Hsl*: F: 5′-ACGCTACACAAAGGCTGCTT-3′ and R: 5′-TCGTTGCGTTTGTAGTGCTC-3′; *Lpl*: F: 5′-CTTTCACTCGGATCCTCTCG-3′ and R: 5′-AGGTGGACATCGGAGAACTG-3′; *Pparγ*: F: 5′-GCCCTTTGGTGACTTTATGGA-3′ and R: 5′-GCAGCAGGTTGTCTTGGATG-3′. qPCR was executed with a QuantStudio 3 (QS3, ThermoFisher Scientific) PCR system. The expression level of the target gene was quantified and normalized to the expression of β-actin. The results are reported as relative gene expression ($2^{-\Delta\Delta CT}$).

Cholesterol and lipid measurement

The cholesterol and triglyceride content of the EDTA-buffered plasma, vWAT (perigonadal), sWAT and liver lysates was measured and quantified using enzymatic assays (Roche Diagnostics; Cat#3039773190 and Cat#4657594190) according to the manufacturer's protocol.

Lipid nanoparticles

The ionisable cationic lipid DLin-MC3-DMA (Hycultec GmbH; Cat#HY-112251) was combined with DSPC (1,2-distearoyl-sn-glycero-3-phosphorylcholine, Avanti; Cat#850365C), Cholesterol (Sigma–Aldrich; Cat#C8667) and DMG-PEG 2000 (Sigma–Aldrich; Cat#880151P) in absolute ethanol at molar ratios of 50:10:38.5:1.5 and a final lipid concentration of 50 mM. The aqueous phase was prepared by dissolving murine small interfering RNA (siRNA)-*Casr* 13.1–3 (Integrated DNA Technologies; sequences: mm.Ri.Casr.13.1: F: 5′-GAACAAAAUCGAUUCUCUGAACCTG-3′ and R: 5′-GUCUUGUUUUAGCUAAGAGACUUGGAC-3'; mm.Ri.Casr.13.2: F: 5′-GACUUCUGGUCCAAUGAGAACCACA-3′ and R: 5′-UACUGAAGACCAGGUUACUCUUGGUGU-3'; mm.Ri.Casr.13.3: F: 5′-ACUGCUUCUGUUGUAAUAGUCCATG-3′ and R: 5′-UUUGACGAAGACAACAUUAUCAGGUAC-3′) in sterile 100 mM acetate buffer (pH 4), to achieve a 0.17 μg/μL solution. For lipid nanoparticle (LNP) formulation, the ethanol and aqueous phase were injected into a microfluidic herringbone mixer (Microfluidic ChipShop) via syringe pumps at a flow rate ratio of 1:3, respectively, with a total

flow rate of 4 mL/min to obtain an N/P ratio of 4. Generated LNPs were diluted with PBS to a concentration of ethanol below 2%, followed by concentrating via ultra-centrifugation (3000×*g*) using 10 kDa Amicon Ultrafilitration units (Sigma–Aldrich; Cat#GE28-9323-60), and were finally dialyzed against PBS using a dialysis membrane with 30 kDa MWCO.

Cell culture

3T3-L1 cells (Sigma–Aldrich; Cat#86052701) were cultured in growth medium (Dulbecco's modified Eagle's medium (DMEM) with high glucose and pyruvate (Sigma–Aldrich; Cat#D6546), 10% foetal calf serum (Bio&Sell; Cat#FBS.ADD.0500) and 1% Penicillin/Streptomycin (Gibco; Cat#15140122)). For differentiation, the cells were seeded in 24-well plates at 80% confluent density in growth medium or differentiation medium.

For differentiation, 80% confluent cell monolayers were incubated for 48 h in differentiation medium (growth medium containing 3-isobutyl-1-methylxanthine (0.5 mM, Sigma–Aldrich; Cat#I5879), insulin (10 µg/ml, Sigma–Aldrich; Cat#I0908) and dexamethasone (1 µM, Sigma–Aldrich; Cat#D2915). Hereafter, adipocytes were kept in post-differentiation medium (growth medium with insulin (2,5 µg/ml)) for an additional 48 h.

Either before or after differentiation, *Casr* knockdown experiments were performed in pre-confluent (80%) 3T3-L1 cells with a final amount of 1 µg of LNPs siRNA-*Casr* or empty LNP per well. The LNPs were incubated for 24 h in growth medium or post-differentiation medium, respectively. Transfection efficiency was measured by qPCR.

Multiplex kinome activity profiling

Tyrosine (PTK) and serine–threonine kinase (STK) profiles were determined using the PamChip® microarray platform with the PamStation®12 (PamGene International). The PTK and STK-PamChip® contain 196 or 144 individual phospho-site(s), respectively. Per array, 10 µg and 2 µg (PTK and STK, respectively) of protein and 400 µM ATP were added. An antibody mix was supplemented to the STK chip to detect the phosphorylated sites. Phosphorylation intensity was quantified (and corrected for local background) using the BioNavigator software (version 6.3, PamGene International). Upstream Kinase Analysis (functional scoring method, PamGene), was used to rank kinases based on combined specificity scores (peptides linked to a kinase, derived from six databases) and sensitivity scores (treatment-control differences). The median final score of the kinase with a score >1.2 and with an adjusted *P*-values for multiple comparisons by the false discovery rate (FDR) of <0.05 are depicted in a heatmap. Pathway enrichment analysis was performed on the differentially expressed kinases (median final scores >1.2 and adjusted *P*-value <0.05) with the use of the R packages clusterProfiler 4.0.[25] The R package disease ontology semantic and enrichment analysis (DOSE)[26] was utilized to analyse the biological complexities in which these kinases correlate with multiple annotation categories, which was visualized in a network plot with the help of the R package Reactive Pathway Analysis (ReactomePA) v1.44.0.[27]

Atherosclerotic lesion induction and analysis

Atherosclerotic lesion formation was analysed in the aortic roots. Hearts were fixated in 4% PFA and embedded in Tissue-Tek O.C.T. compound (Sakura) for transverse cryo-sectioning (4 µm). Atherosclerotic lesion sizes were quantified after H&E (Sigma–Aldrich) staining and averages were calculated from 3 sections per mouse. Sections were imaged using a Leica DMLB microscope and charge-coupled device (CCD) camera. Analyses were performed blinded with the use of the QuPath-0.3.2 software.

Genetic association analysis of CASR variants related to BMI

Data from multiple genome-wide association studies (GWAS) in the cardiovascular and metabolic field were accessed via the Common Metabolic Disease and Knowledge Portal (CMDKP, https://hugeamp.org, accessed 2023/08/18). We specifically scanned the *CASR* locus (±50 kb) for variants associated with body mass index in all ancestries and the genetic association was computed by meta-analysis pooling data across multiple cohorts (effective sample size 3,825,200) by using the bottom-line methods implemented in METAL and available through CMDKP.[28] The association between the *CASR* locus and the prevalence of coronary artery disease was studied on the latest meta-analysis of the Coronary ARtery DIsease Genome-wide Replication and Meta-analysis (CARDIoGRAM) plus the Coronary Artery Disease (C4D) including 210,842 cases among 1,378,170 participants.[29] The functional relevance of the variant for transcription factor binding was estimated on position weight matrices (PWMs) in ENCODE TF binding experiments determined by Kheradpour and Kellis.[30]

Statistics

All data are expressed as mean ± standard error of mean (SEM). Statistical analyses were performed using GraphPad Prism 9 or higher (GraphPad Software Inc.). After identifying outliers via the ROUT method, normal distribution was verified with the Shapiro–Wilk normality test. Unpaired Student's *t* test with Welch's correction or Mann–Whitney *U* test were performed afterward, as appropriate. The areas under the curve (AUCs) for the GGT and ITT tests were compared by two-way ANOVA with Bonferroni *post hoc* test. Pairwise correlation between plaque burden and plasma lipids was calculated by Spearman's correlation test. A two-tailed *P* value < 0.05 was considered statistically significant.

Role of funders
The funders did not have any role in study design, data collection, data analysis, interpretation, or writing of this manuscript.

Results

Female mice are obesity resistant, an effect that is eradicated upon adipocyte-*Casr* deficiency

To study the effect of sex and the role of adipocyte CaSR in obesity we crossbred male and female *AdipoqCre* and *Casr*^{flox} mice. After tamoxifen injections, to induce the deficiency, and AAV8-PCSK9 injection, to induce hyperlipidaemia, mice were subjected to a 12-week DIO diet after which various parameters were evaluated (Fig. 1a). As reported for HFD induced obesity mouse models,[8,9] a sex-difference was observed as female mice were rather resistant to the obesogenic effects of the DIO diet characterized by a lack of body weight gain over time, while male mice showed a rapid increase in body weight (Fig. 1b). Although female mice already had a slight but significantly reduced body weight at baseline, at the end of the experiment the females had a strikingly lower body weight compared to male mice (Fig. 1c–d). Interestingly, female adipocyte-specific *Casr*-deficient (*AdipoqCre*$^{+}$*Casr*^{flox}) mice gained significantly more body weight, compared to control (*Casr*^{flox}) female mice, while male adipocyte-specific *Casr*-deficient mice demonstrated similar body weight gain as their sex-matched controls (Fig. 1b–d). Similar sex-specific differences were also observed in the visceral white adipose tissue (vWAT) weight. This sex-difference was abolished upon adipocyte-specific *Casr* deficiency (Fig. 1e). However, no changes in liver weight could be observed (Fig. 1f). Furthermore, plasma PTH as well as PTH and calcium levels in vWAT were unchanged (Supplemental Fig. S1a-c). Histological analysis confirmed that the vWAT of female mice contained smaller adipocytes compared to male mice, characterized by a reduced surface and diameter, while this sex-difference was again abolished upon adipocyte-specific *Casr* deficiency (Fig. 1g–i). In line with these results, it could be observed that female mice were less glucose intolerant and insulin resistant, compared to male mice. This sex-difference was at least partly diminished again upon adipocyte-specific *Casr* deficiency (Fig. 1j–m). Overall, these results clearly demonstrate that female mice are more resistant for obesity development compared to males, which is a phenomenon that is eradicated when CaSR is lacking in adipocytes.

To assess the possible translational relevance of our results to humans, we have performed a bottom-line integrative analysis of genetic association studies for variants in the *CASR* locus (chr3: 121.852.515–122.060.476). We found that the single nucleotide polymorphism (SNP) rs7648041 (global minor allele frequency (MAF) 27.6%) was significantly associated with body mass index (BMI) in humans ($P < 3.4 \times 10^{-6}$), showing wide consistency across the largest cohorts (Supplemental Fig. S2 and Table S1). Functionally, rs7648041 is upstream of *CASR*, and overlaps with binding motifs for the transcription factors PU.1 and TCF4, which is predicted to affect their binding (Supplemental Table S2) as well as to disrupt a vitamin D response element.[31] Together, these data reveal that a genetic variant that is likely affecting *CASR* expression is associated with BMI, suggesting a role of this receptor in regulating adiposity also in humans.

Females show an adipocyte-specific *Casr* dependent elevated vWAT inflammation compared to males

Since obesity results in dysfunctional AT, characterized by elevated inflammation,[32] cytokine levels were measured in vWAT lysates. Strikingly, while the body and vWAT weight in female mice was significantly smaller compared to males, the vWAT of female mice contains a significantly higher amount of pro-inflammatory cytokines tumour necrosis factor (TNF) and interleukin 6 (IL-6) (Fig. 2a–b), while the chemokine CC-chemokine ligand 2 (CCL2) does not show any sex-differences (Fig. 2c). These sex-differences in TNF and IL-6, are completely eradicated upon adipocyte-specific *Casr*-deficiency (Fig. 2a–b), while adipocyte-specific *Casr*-deficiency results in a significant reduction of CCL2 expression in vWAT which is sex unspecific (Fig. 2c). Similar effects are also observed in subcutaneous WAT (sWAT), while in this depot CCL2 also shows sex-differences (Supplemental Fig. S3a–c). In order to evaluate whether this elevated inflammation also coincided with an elevated leukocyte infiltration, flow cytometry was performed on vWAT demonstrating that mainly CD3^{+} T cell, and more specifically CD3^{+}CD8^{+} cytotoxic T cell counts, were significantly elevated in female, compared to male mice. This sex-difference was abrogated upon adipocyte-specific *Casr*-deficiency (Fig. 2d–i).

Inflammation in the liver was also evaluated, since the liver is also affected by obesity.[33] In line with the vWAT and sWAT results, TNF in the liver was significantly higher in female mice compared to male mice (Supplemental Fig. S3d), while IL-6 and CCL2 only demonstrated a slight but consistent trend (Supplemental Fig. S3e and f). The sex effect in the liver on TNF was again inverted upon adipocyte-specific *Casr* deficiency (Supplemental Fig. S3d).

Together, these results clearly demonstrate that female mice have elevated vWAT, sWAT and hepatic inflammation compared to male mice, while adipocyte-specific *Casr* deficiency eradicates this sex effect on inflammation.

WAT from females shows a CaSR-dependent higher expression of lipid uptake and lipolysis genes

Previous studies revealed that the ERα plays an important role in adiposity in mice[12,13] and that the activity of

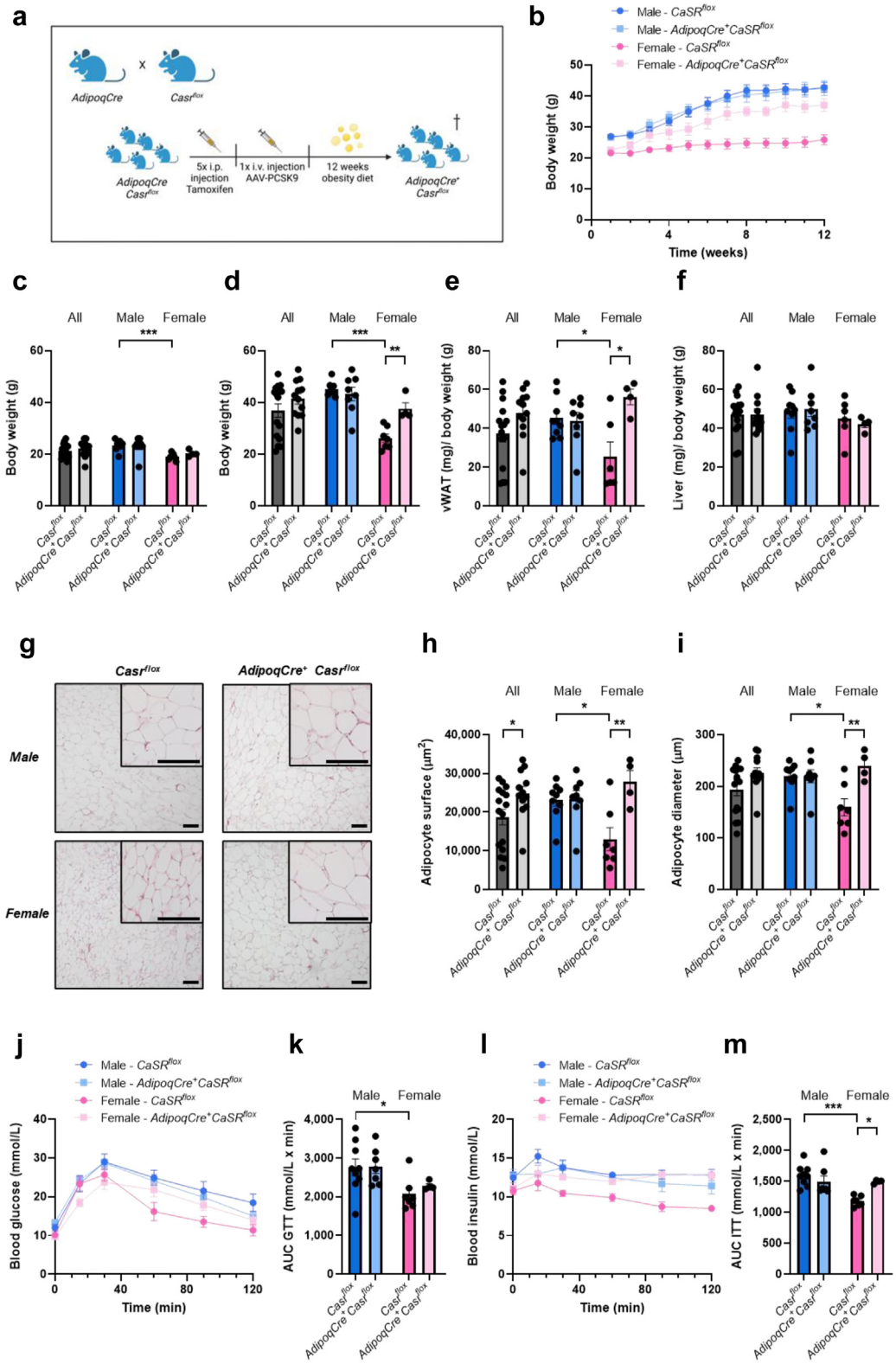

Fig. 1: **Female mice show reduced body and adipose tissue weight and improved glucose and insulin handling compared to male mice, while adipocyte-specific *Casr* deficiency eliminates these sex-differences.** (**a**) Representation of workflow. Induction of knockout by i.p.

this receptor is affected by the CaSR.[14] Therefore, we evaluated whether the expression of ERα is affected in our animal model. Female mice had a significantly elevated expression of *Erα*, while this sex-difference was only marginally affected by the adipocyte-specific *Casr*-deficiency (Fig. 3a). In line with this, it could be confirmed *in vitro* that knock-down of *Casr* in adipocytes results in a significantly reduced *Era* expression (Supplemental Fig. S4a). Oestrogen levels were not changed in the vWAT or plasma of the mice, suggesting that the observed effects are receptor rather than ligand mediated (Supplemental Fig. S4b and c). Since oestrogens and the ERα have been shown to affect various adipocyte functions, like lipid handling at least in part via peroxisome proliferator-activated receptor γ (PPARγ),[34] we also evaluated the effects on PPARγ signalling and other genes that play a key role in lipid metabolism in vWAT. Interestingly, female mice indeed also have a higher expression of *Pparγ* (Fig. 3b) and its downstream target genes adipocyte protein 2 (*aP2*), lipoprotein lipase (*Lpl*) and CCAAT/enhancer binding protein α (*C/ebpα*) compared to males (Fig. 3c–e). Remarkably, a previous study by He et al. reported that CaSR stimulation of human SW872 adipocytes *in vitro* increased the gene expression of PPARγ and its downstream targets.[22] These observations are in line with our model as adipocyte-specific *Casr* deficiency significantly reduces *Pparγ* and *aP2* expression and results in a trendwise reduction of *Lpl* expression *in vivo* in female mice (Fig. 3b–d). PPARγ and aP2 are involved in adipocyte differentiation and adipogenesis, suggesting that these processes are also reduced in female mice lacking adipocyte *Casr*. This notion is further supported by the observation that knock-down of *Casr* in pre-adipocytes during the differentiation process results in a significant reduction of adipocyte differentiation *in vitro*, characterized by reduced *Pparγ*, *aP2* and *Adipoq* expression (Supplemental Fig. S5a–c). Furthermore, activation of the ERα in skeletal muscle increases the expression of CD36,[35] a major lipid uptake receptor and downstream target of PPARγ. Strikingly, *Cd36* expression was significantly elevated in female, compared to male mice, while an adipocyte-specific *Casr* deficiency again diminished this expression (Fig. 3f). Besides lipid uptake, we could also observe sex-differences in expression levels of genes related to lipolysis, as the expression of *Atgl* and *Hsl* was significantly higher in female, compared to male mice (Fig. 3g–h). However, the expression was only marginally reduced in female adipocyte-specific *Casr* deficient mice (Fig. 3g–h), implying that the absence of adipocyte CaSR did not majorly affect lipolysis.

These results clearly indicate that there are major sex-differences in genes related to lipid metabolism in vWAT that are at least partly mediated by the adipocyte CaSR. To determine whether the lipid accumulation is also affected by these sex-differences and by the lack of adipocyte-specific *Casr*, cholesterol and triglyceride levels were measured in the vWAT. Although we could not observe a clear sex-difference in the control mice, the cholesterol and triglyceride content in vWAT of particularly female mice was significantly reduced upon adipocyte-specific *Casr* deficiency (Fig. 3i–j). Although less pronounced, similar effects could also be observed in sWAT (Supplemental Fig. S6a–j).

These data suggest that female mice show a high expression of lipid uptake genes which is balanced by an elevated expression of lipolysis genes, that combined do not influence the total vWAT lipid levels. Interestingly, this balance shifts upon adipocyte-specific *Casr* deficiency, as gene expression levels as well as vWAT lipid levels are reduced in female mice lacking adipocyte CaSR.

Inflammatory signalling pathways are more activated in females in a CaSR dependent manner

In order to pinpoint the underlying mechanisms behind the observed sex-differences in vWAT, an unbiased multiplex kinome activity profiling was performed measuring the activity of tyrosine and serine–threonine kinases in vWAT lysates. Female mice demonstrated a significantly increased activity of various inflammation-related serine–threonine kinases like Akt, 5' AMP-activated protein kinase (AMPK) and several protein kinase C (PKC) isoforms compared to male mice (Fig. 4a; top row), while tyrosine kinase activity was rather unaffected (Fig. 4b; top row). Interestingly, there is a clear reduction of cyclin-dependent kinases (CDKs) in female mice, compared to male mice (Fig. 4a; top row), which might explain the observed reduced relative vWAT weight in female mice and is consistent with a reduced adipocyte differentiation. In line with the cytokine results, various inflammation-related kinases were again significantly downregulated in female mice upon adipocyte-specific *Casr*-deficiency in comparison to control mice, e.g., Akt, AMPK, various PKC isoforms

injection of tamoxifen for 5 consecutive days, followed by AAV-PCSK9 i.v. injection and 12 weeks obesity-inducing diet (DIO) feeding. (**b–d**) Quantification of (**b**) body weight gain over time, (**c**) baseline body weight and (**d**) end-point body weight (n = 8–9 for males; n = 4–7 for females). (**e–f**) Quantification of (**e**) vWAT weight (n = 8–9 for males; n = 4–6 for females) and (**f**) liver weight (n = 8–9 for males; n = 4–6 for females) normalized to total body weight. (**g–i**) Analysis of vWAT by (**g**) HE staining (Scale bar = 10 μm) and quantification of (**h**) adipocyte surface and (**i**) adipocyte diameter (n = 8–9 for males; n = 4–7 for females). (**j–m**) Blood glucose levels during (**j and k**) a (**j**) glucose tolerance test (GTT) with (**k**) quantification of the AUC as well as (**l and m**) an (**l**) insulin tolerance test (ITT) with (**m**) quantification of the AUC (n = 7–9 for males; n = 4–7 for females). Graphs represent mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001. AUC: area under the curve.

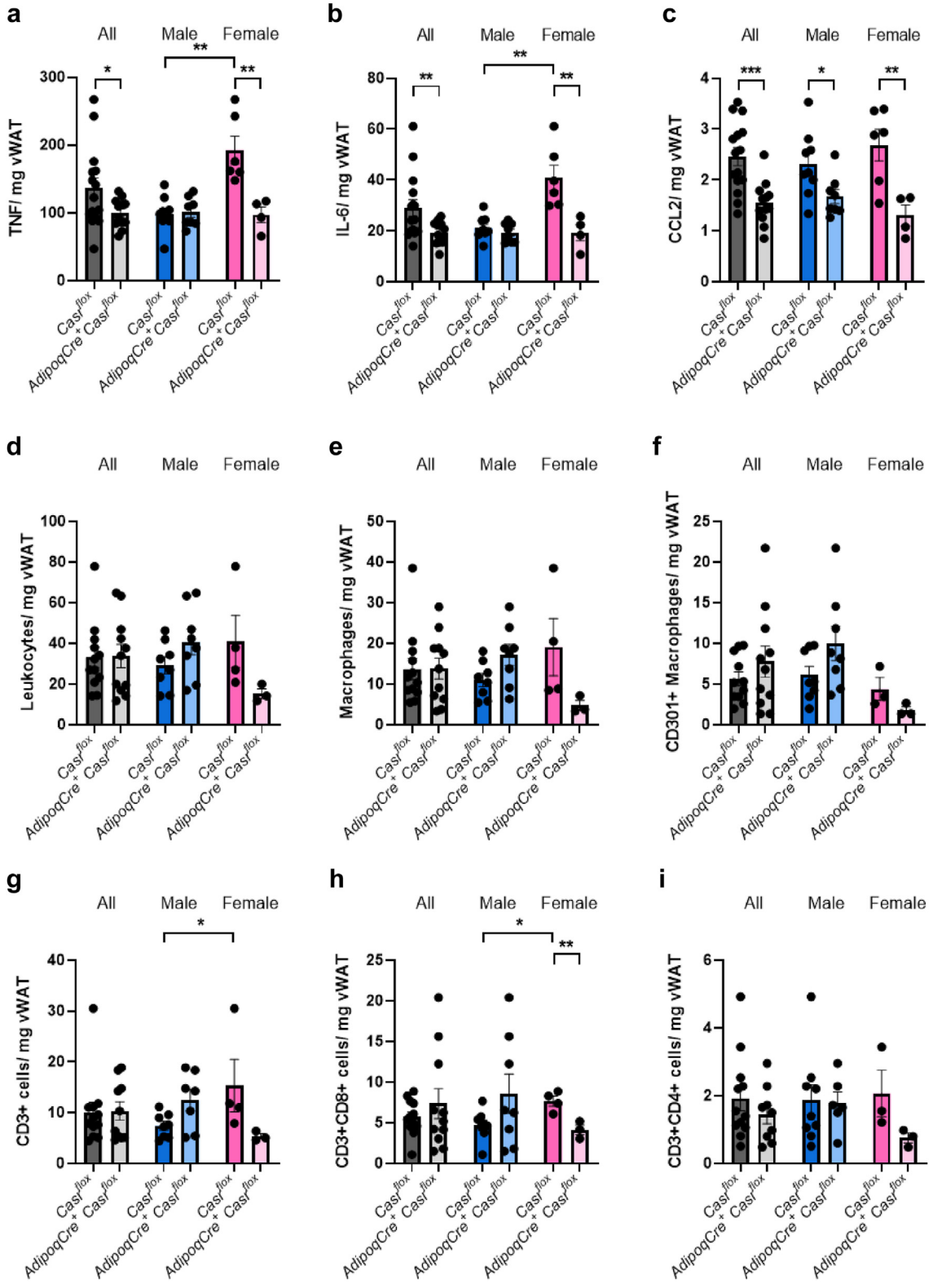

Fig. 2: **Female mice demonstrate elevated vWAT inflammation and T cell infiltration, compared to male mice.** (**a–c**) Quantification of inflammatory cytokines (**a**) TNF (n = 8–9 for males; n = 4–6 for females), (**b**) IL-6 (n = 8–9 for males; n = 4–6 for females) and (**c**) CCL2 (n = 8–9

(Fig. 4a; bottom row) as well as Src and Syk (Fig. 4b; bottom row).

Detailed pathway analysis revealed that the significantly sex-specific (control females vs. control males) regulated kinases play a key role in general inflammatory pathways, like PI3K-Akt signalling and TNF signalling (Table 1) and also particularly in T cell related signalling cascades, in line with the observed accumulation of T cells in vWAT in female compared to male mice (Table 1 and Supplemental Tables S3–S8). Strikingly and consistent with the notion that calcium as well as oestrogen receptor signalling are influenced by sex, the significantly regulated kinases also play important role in these signalling cascades characterized by pathways like calcium signalling, calcium ion transport and oestrogen signalling pathway (Table 1).

Sex-differences in plasma lipid levels and atherosclerosis are diminished upon *Casr* deficiency

In addition to local effects, WAT inflammation can also have systemic/vascular effects and is therefore also related to cardiovascular pathologies, like atherosclerosis.[36,37] Therefore, we were curious if adipocyte-specific *Casr* deficiency also had systemic effects. Circulating lipid levels were evaluated, and strikingly, cholesterol as well as triglyceride levels were both reduced by 71% in female compared to male mice (Fig. 5a–b). Adipocyte-specific *Casr* deficiency at least partly reversed these sex-differences as plasma cholesterol and triglyceride levels were elevated in these *Casr*-deficient mice (Fig. 5a–b). Liver lipid levels demonstrated, at least for triglycerides, a similar pattern as triglycerides were reduced in female compared to male mice, but elevated again upon *Casr*-deficiency (Supplemental Fig. S7 and b). Additionally, the systemic effects on cardiometabolic diseases, more particularly atherosclerotic plaque formation were analysed. Female mice almost developed no atherosclerosis which was in sharp contrast to male mice (Fig. 5c). In line with the elevated circulating lipid levels, an adipocyte-specific knockout of *Casr* resulted in significantly larger atherosclerotic plaques in female mice, which are in size comparable to male mice (Fig. 5c). Furthermore, in accordance with the effects on circulating lipids, plaque sizes in males were not affected by the lack of adipocyte *Casr* (Fig. 5c). There is a strong correlation between the circulating cholesterol and triglyceride levels and atherosclerotic plaque size (Fig. 5d–e), suggesting that these circulating lipid levels are the driving force behind the observed effects on atherosclerosis development in our mouse model. Finally, screening of a meta-analysis of genome-wide association studies including data from 1,378,170 participants and 210,842 coronary artery disease (CAD) cases revealed the association of the *CASR* locus with CAD with the lead SNP (i.e., rs17251221) achieving a significance level approximating a 1% FDR ($P = 1.1 \times 10^{-5}$).[29] These results therefore demonstrate that female mice are highly protected against obesity-associated atherosclerosis in an adipocyte-specific *Casr* dependent manner.

Discussion

Previous studies have demonstrated that female mice are more resistant to the obesogenic effects of HFD, compared to male mice.[8,9] However, the underlying mechanisms remained rather elusive especially since many obesity studies so far did not focus on sex-differences but rather selected males for their studies. Therefore, in this study we set out to elucidate the mechanisms behind sex-differences in the context of HFD-induced obesity, particularly focusing on the role of the CaSR.

In line with earlier observations, we could demonstrate that females are resistant to HFD-induced obesity as they did not gain as much weight over time, resulting in lower body and vWAT weight, characterized by smaller adipocytes. Interestingly, this effect was completely abolished upon adipocyte-specific *Casr* deficiency, clearly demonstrating that the CaSR plays an important role in the observed sexual dimorphisms. This dimorphism might be explained by sex hormones, as an expansion of vWAT coincides with increased CYP19A1 expression, an aromatase that converts androgens into oestrogen, leading to higher oestrogen levels. This in turn could affect lipid metabolism as aromatase deficiency has been shown to increase triglyceride levels and fatty liver disease.[10] Furthermore, Ren et al. demonstrated that testosterone inhibits preadipocyte differentiation by switching macrophages from M1 to M2 polarization through the $G\alpha_i$ and Akt signalling pathways.[38] Additionally, animal models have shown that sex hormones influence adipocyte development, lipolysis, as well as the inflammatory phenotype in response to obesity.[10] Indeed, we observed that female mice express a high level of *Erα*, compared to male mice, while this difference was sharply reduced upon adipocyte-specific *Casr* deficiency. Previously, it could already be demonstrated that deletion of the *Erα* in adipocytes resulted in an increased adiposity in mice,[12,13] while CaSR was shown play a role in ERα activity *in vitro*.[14] In this study we confirmed in 3T3-L1 adipocytes that *Casr* deficiency results in a decreased *Era* expression *in vitro*. Combined, this suggests that the ERα is at least partly responsible for the observed effects

for males; n = 4–6 for females) in vWAT normalized to 1 mg vWAT. (**d–i**) Quantification of leukocyte presence in vWAT analysed using flow cytometry, showing (**d**) total leukocyte, (**e**) macrophage, (**f**) $CD301^+$ macrophage, (**g**) $CD3^+$ T cell, (**h**) $CD3^+CD8^+$ T cell and (**i**) $CD3^+CD4^+$ T cell counts. Graphs represent mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. vWAT: visceral white adipose tissue.

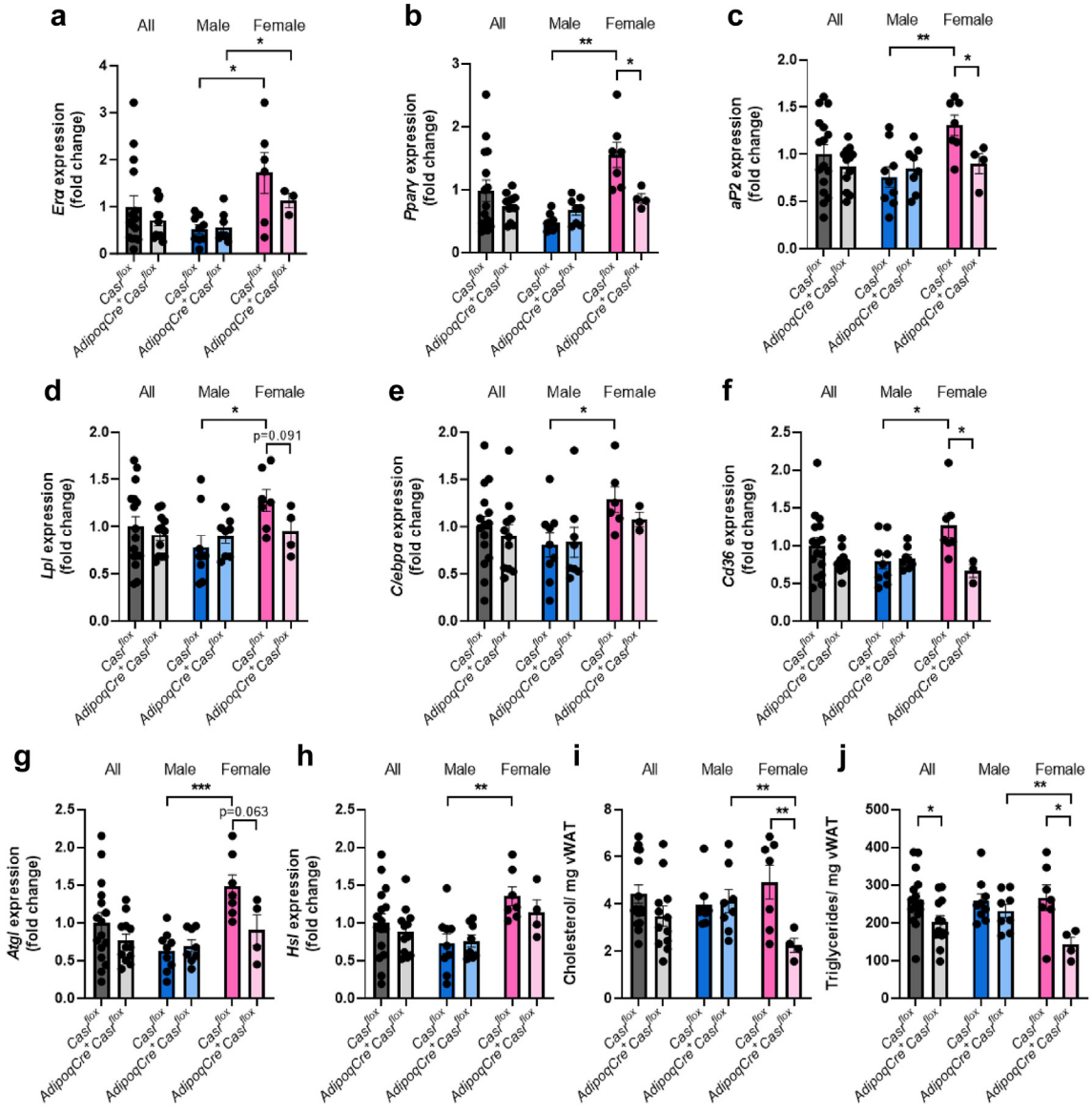

***Fig. 3:* Adipocyte-specific *Casr* deficiency reduces expression of genes related to adipocyte differentiation and adipogenesis in vWAT.** Fold change of gene expression of (**a**) oestrogen receptor α (*Era*), (**b**) peroxisome proliferator-activated receptor γ (*Pparγ*), (**c**) adipocyte protein 2 (*aP2*), (**d**) lipoprotein lipase (*lpl*), (**e**) CCAAT/enhancer binding protein α (C/ebpa), (**f**) cluster of differentiation 36 (*Cd36*), (**g**) adipocyte triglyceride lipase (*Atgl*) and (**h**) hormone-sensitive lipase (*Hsl*) in vWAT (n = 8–9 for males; n = 3–7 for females). (**i and j**) Quantification of (**i**) total cholesterol and (**j**) triglyceride content in vWAT lysates (normalized to vWAT weight; n = 8–9 for males; n = 4–7 for females). Bar graphs represent mean ± SEM. **P* < 0.05; ***P* < 0.01; ****P* < 0.001. vWAT: visceral white adipose tissue.

on vWAT and body weight in female mice, which is also supported by the kinase activity profiling showing clear effects on oestrogen related signalling pathways.

Although the weight of the vWAT was lower in female mice, it was characterized by an elevated amount of tissue cytokines TNF and IL-6, while CCL2 was not affected by sex. This effect was completely CaSR dependent as adipocyte-specific *Casr* deficiency significantly reduced the cytokine levels, thereby diminishing the sex-differences. Previous studies have demonstrated an important role for CaSR in AT inflammation *in vitro* using pharmacological targeting.[21,22] It has been demonstrated that pharmacological activation of the CaSR results in an elevated expression of pro-inflammatory IL-6, TNF, and CCL2 in an adipocyte cell line derived from human liposarcoma (LS14).[39] Furthermore, CaSR activation in LS14 cells was seen to activate the NLR family pyrin domain containing 3,

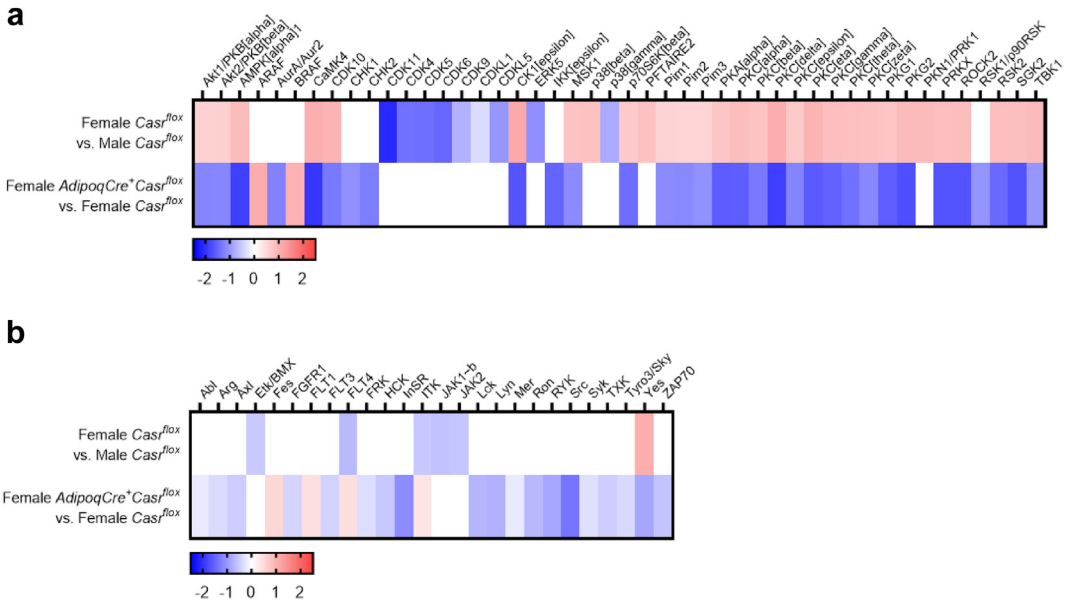

Fig. 4: **Inflammatory kinase activity in the vWAT is upregulated in female mice in a CaSR dependent manner.** (**a**) Serine–threonine kinase (STK) and (**b**) Phosphotyrosine kinase (PTK) activity in the vWAT. Only significantly regulated kinases are shown. (**a and b**) Top rows show sex-differences, where red indicates increased and blue decreased kinase activity in female compared to male control mice. Bottom rows show differences between female adipocyte-specific *Casr* deficient mice compared to female control mice, where red indicates increased and blue decreased kinase activity. vWAT: visceral white adipose tissue. Presented data is an average of 4 individual samples per genotype and per gender.

which promotes the secretion of IL-1β, and furthermore increases autophagy, which leads to enhanced TNF secretion.[40,41] In accordance with these *in vitro* findings, the current study showed that adipocyte-specific CaSR also enhances pro-inflammatory cytokine levels in vWAT *in vivo*, fuelling local inflammation. These elevated cytokine levels coincided with an elevated accumulation of T cells, particularly $CD8^+$ T cells in the vWAT. Previous studies already clearly demonstrated that $CD8^+$ T cells play an important role in the initiation and maintenance of AT inflammation, characterized by elevated levels of IL-6 and TNF.[42,43] Therefore, it is plausible that this elevated T cell accumulation is responsible for the elevated cytokine levels in vWAT in our study. This is in contrast to our previous study using an atherosclerotic $Apoe^{-/-}$ mouse model fed with a Western-type diet (21% fat and 0.15–0.2% cholesterol) in which adipocyte-specific *Casr* deficiency did not affect inflammation of the vWAT.[23] Therefore, the observed effects seem to be diet-dependent and occur only in a condition of HFD-induced obesity.

Since vWAT is a major organ for lipid deposition, we also studied the effect of sex and adipocyte-specific *Casr* deficiency on lipid metabolism. In line with other studies, we could demonstrate that the expression of *Pparγ* and its downstream target genes was elevated in the vWAT of female mice, which coincided with a high expression of *Erα*.[34,44] PPARγ is known to play a crucial role in the regulation of adipocyte differentiation and adipogenesis together with C/EBPα.[45,46] Furthermore, as reported by previous *in vitro* studies,[22] it could be confirmed *in vivo* that CaSR influences *Pparγ* expression. A previous *in vitro* study demonstrated that CaSR activation in SW872 adipocytes results in an upregulation of PPARγ, resulting in increased adipocyte differentiation and expression of *aP2*, which plays an important role in fatty acid metabolism.[22] Moreover, activation of PPARγ promotes the expression of genes involved in lipid uptake, such as *Cd36*.[47] In line with these observations, we also observed a higher *Cd36* expression in female compared to male mice, in an adipocyte-*Casr* dependent manner. Together with the notion that the lipid content in vWAT from particularly female mice lacking adipocyte-specific *Casr* was strongly decreased, compared to controls, it can be suggested that especially lipid uptake driven by changes in *Pparγ* is affected. Previous *in vitro* studies have shown that upregulation of CaSR does not only enhance lipid uptake but is also responsible for impaired lipolysis, through downregulation of catabolite activator protein (cAMP).[48] However, in our *in vivo* model we primarily observed sex-specific effects on genes involved in lipolysis (*Atgl* and *Hsl*), while these effects were only moderately dependent on CaSR in which the adipocyte-specific *Casr* deficiency actually slightly reduced the gene expression of *Atgl* and *Hsl*. It should however be noted that the *in vitro* studies only measured the expression of lipolysis related genes after

Description	ID	Adjusted *P* value
Inflammation		
Rap1 signalling pathway	hsa04015	4.65E-06
PI3K-Akt signalling pathway	hsa04151	1.78E-05
PI3K-Akt signalling pathway	WP4172	6.08E-05
TNF signalling pathway	hsa04668	1.49E-04
Positive regulation of NF-kappaB transcription factor activity	GO:0051092	6.48E-04
I-kappaB kinase/NF-kappaB signalling	GO:0007249	9.62E-04
TNF-alpha signalling pathway	WP231	1.96E-02
Regulation of inflammatory response	GO:0050727	3.87E-02
T cell		
T cell receptor signalling pathway	hsa04660	9.36E-06
Alpha-beta T cell activation	GO:0046631	1.51E-02
T cell receptor and co-stimulatory signalling	WP2583	1.83E-02
T-cell receptor signalling pathway	WP69	1.88E-02
Positive regulation of T cell activation	GO:0050870	2.57E-02
Regulation of T cell activation	GO:0050863	2.65E-02
Positive regulation of alpha-beta T cell proliferation	GO:0046641	4.05E-02
T cell receptor and co-stimulatory signalling	WP2583	4.36E-02
Regulation of alpha-beta T cell proliferation	GO:0046640	4.89E-02
Alpha-beta T cell proliferation	GO:0046633	4.97E-02
Oestrogen		
Thyroid hormone signalling pathway	hsa04919	1.88E-05
Endocrine and other factor-regulated calcium reabsorption	hsa04961	1.13E-04
Thyroid hormone synthesis	hsa04918	4.00E-04
Parathyroid hormone synthesis, secretion and action	hsa04928	1.21E-03
Oestrogen signalling pathway	hsa04915	2.93E-03
Oestrogen signalling pathway	WP712	1.35E-02
Calcium		
Endocrine and other factor-regulated calcium reabsorption	hsa04961	1.72E-05
Calcium signalling pathway	hsa04020	5.02E-04
Regulation of calcium ion transmembrane transport	GO:1903169	2.19E-02
Calcium ion transport	GO:0006816	4.39E-02
Regulation of release of sequestered calcium ion into cytosol	GO:0051279	4.56E-02

Table 1: **Selection of signalling pathways focusing on inflammation, T cells, oestrogen, and calcium.**

calcium stimulation and additionally it should be taken into consideration that the processes *in vivo* are obviously more complex, which might explain this discrepancy.[49]

Focusing on the underlying signalling cascades, female mice were shown to have reduced activity of CDKs, compared to male mice, which might be at least partly responsible for the reduced vWAT weight as CDKs play an important role in adipocyte differentiation.[50] So far there is no literature available regarding sex-differences in CDK activity in adipose tissue, making this an interesting topic for future studies, particularly since we could demonstrate that these effects do not seem to be dependent on CaSR. On the other hand, female mice showed a strongly increased activity of various inflammation-related kinases, which does seem to be very dependent on CaSR. One of the most affected signalling cascades was NF-κB, which has already been shown to be upregulated in dysfunctional AT.[1,51,52] Consequently, NF-κB, a transcription factor, plays a vital role in the regulation of inflammatory factors, such as IL-6, TNF and CCL2. Interestingly, NF-κB can interact with the 5′region of the *Casr* gene which proposes the involvement of CaSR in exacerbating the pro-inflammatory environment in the vWAT.[51] This observation is in line with our results, where the lack of adipocyte-specific *Casr* leads to a reduction in inflammation of the vWAT and, additionally downregulates the kinases involved in the NF-κB signalling pathway. However, it should be noted that only upstream signalling pathways were studied, making NF-κB activation and translocation as a downstream target, an interesting field for upcoming studies. Additionally, our kinome profiling showed that the observed sex-differences also affect T cell related signalling as well as oestrogen related pathways, further supporting the notion that these pathways play an important role in the observed sex-as well as CaSR-differences.

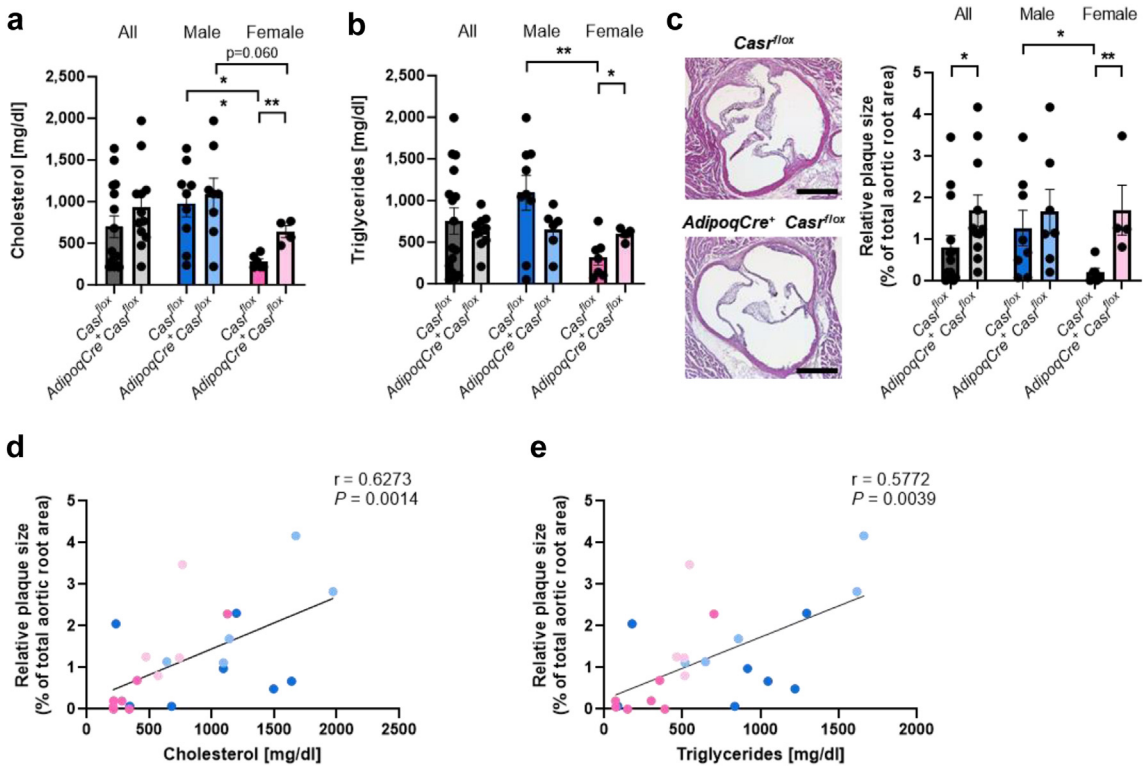

Fig. 5: **Female mice are protected against obesity-associated atherosclerosis in an adipocyte-specific *Casr* manner.** (**a–b**) Blood plasma lipid levels of (**a**) cholesterol (n = 8–9 for males; n = 4–6 for females) and (**b**) triglycerides (n = 8–9 for males; n = 4–7 for females). (**c**) Representative images of H&E-staining in the aortic root of $Casr^{flox}$ and $AdipoqCre^{+}Casr^{flox}$ mice and quantification of relative plaque size (% of total aortic root area) (n = 7–8 for males; n = 4–6 for females). Scale bar: 500 µm. (**d–e**) Correlation analysis of plasma lipid levels and atherosclerotic plaque size (n = 5–7 for males; n = 4–7 for females; colour codes correspond with bar-graphs in panels A–C). Bar graphs represent mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$.

We have observed lower circulating lipid levels in female, compared to male mice, which is in line with the notion that female mice are more resistant to the obesogenic effects of the HFD as also demonstrated by the reduced vWAT content. This is also in line with previous studies showing that female mice have a reduced weight gain over time[8] and have a greater ability to use dietary fat as energy source.[9] In accordance with the reduced lipid uptake by the vWAT in mice lacking adipocyte-specific *Casr*, an elevation in cholesterol and triglycerides levels could be observed in their circulation. This elevation in circulating lipids coincided also with elevated hepatic triglyceride levels, at least suggesting that there is elevated lipid accumulation in the liver which may explain the observed effects on circulating lipids. Since circulating lipids are a major driving factor for cardiometabolic diseases, such as atherosclerosis,[53] we were interested to investigate the effects on atherosclerotic plaque formation. While several studies show that female mice generally have larger atherosclerotic lesions, compared to male mice,[54,55] in our study we have observed that female mice are almost completely protected against atherosclerosis development. An important notion and probable explanation for this discrepancy is the fact that classical atherosclerosis models are based on Western-type or high-cholesterol diet feeding to induce atherosclerosis, while we have used a HFD to induce obesity and obesity-associated atherosclerosis. Furthermore, our study revealed that adipocyte-specific *Casr* deficient mice have enhanced plaque formation in comparison to control mice, thereby completely abolishing the sex-effect. These effects may be explained by the elevation of plasma lipid levels in these mice as these were strongly correlated with plaque size. Since circulating lipids also play an important role in other cardiometabolic diseases, like myocardial infarction or stroke, it would, in the future, be interesting to also investigate the effects of sex and adipocyte-specific *Casr* deficiency on other pathological models.

In conclusion, CaSR seems to more actively promote WAT dysfunction in female mice, compared to male mice. This sex effect could be abolished for most parameters upon adipocyte-specific *Casr* deficient in

female mice, making them again comparable to male mice. It is plausible that the interaction of CaSR with the ERα plays an important role in these dimorphic effects, as *Erα* expression as well as oestrogen-related signalling pathways are affected in adipocyte-specific *Casr* deficient mice. However, it cannot be excluded that differences in circulating ligands of the CaSR are at least partly responsible for the observed effects, which therefore remains a focus point for future research. Although the local WAT effect would suggest that CaSR inhibition would be a promising target to treat WAT dysfunction especially in females, the resulting systemic elevation in lipid levels and atherosclerosis formation warrant caution for such approaches. Future studies are needed to elucidate whether CaSR expression on other adipose tissue related cells (e.g., immune cells) also play a role in vWAT inflammation, perhaps without affecting the systemic lipid levels that fuel cardiometabolic diseases.

Contributors

Conceptualization: S.L.M., J.J., E.A.L.B. and E.P.C.v.d.V.
Data curation: S.A., S.L.M., R.H., L.R., K.A., M.T., K.W. and E.P.C.v.d.V.
Formal analysis: S.A., S.L.M., D.S. and E.P.C.v.d.V.
Funding acquisition: E.P.C.v.d.V.
Project administration: E.P.C.v.d.V.
Resources: M.H. L.L. and M.B.
Supervision: J.J., E.A.L.B. and E.P.C.v.d.V.
Visualization: S.A., S.L.M. and E.P.C.v.d.V.
Roles/Writing - original draft: S.A.
Verified the underlying data: S.L.M. and E.P.C.v.d.V.
Writing - review & editing: J.J., S.L.M., E.A.L.B. and E.P.C.v.d.V.
All authors read and approved the final version of the manuscript.

Data sharing statement

The complete overview of the pathway analyses is included as Supplemental Tables S3–S8. Other raw data, will be made available to interested readers upon reasonable request.

Declaration of interests

None.

Acknowledgements

This work was supported by a grant from the Interdisciplinary Center for Clinical Research within the faculty of Medicine at the RWTH Aachen University and by the Corona Foundation (S199/10084/2021) to E.P.C.v.d.V., by the Deutsche Forschungsgemeinschaft (DFG) (SFB TRR219 – Project-ID 322900939; subproject M02 to M.H. and M07 to E.P.C.v.d.V. and SFB1123-B05 to D.S.) and the BMBF and Free State of Bavaria (LMU Excellence strategy 80325065) and the DZHK 81Z0600203 and 81X2600269 to D.S.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2024.105293.

References

1 van der Heijden RA, Sheedfar F, Morrison MC, et al. High-fat diet induced obesity primes inflammation in adipose tissue prior to liver in C57BL/6j mice. *Aging (Albany NY)*. 2015;7(4):256–268.
2 Finucane MM, Stevens GA, Cowan MJ, et al. National, regional, and global trends in body-mass index since 1980: systematic analysis of health examination surveys and epidemiological studies with 960 country-years and 9.1 million participants. *Lancet*. 2011;377(9765):557–567.
3 Knopp RH, Paramsothy P, Retzlaff BM, et al. Gender differences in lipoprotein metabolism and dietary response: basis in hormonal differences and implications for cardiovascular disease. *Curr Atheroscler Rep*. 2005;7(6):472–479.
4 Menegoni F, Galli M, Tacchini E, Vismara L, Cavigioli M, Capodaglio P. Gender-specific effect of obesity on balance. *Obesity*. 2009;17(10):1951–1956.
5 Gerdts E, Regitz-Zagrosek V. Sex differences in cardiometabolic disorders. *Nat Med*. 2019;25(11):1657–1666.
6 Regitz-Zagrosek V, Lehmkuhl E, Mahmoodzadeh S. Gender aspects of the role of the metabolic syndrome as a risk factor for cardiovascular disease. *Gend Med*. 2007;4(Suppl B):S162–S177.
7 Woitowich NC, Beery A, Woodruff T. A 10-year follow-up study of sex inclusion in the biological sciences. *Elife*. 2020;9:e56344.
8 Casimiro I, Stull ND, Tersey SA, Mirmira RG. Phenotypic sexual dimorphism in response to dietary fat manipulation in C57BL/6J mice. *J Diabetes Complications*. 2021;35(2):107795.
9 Oraha J, Enriquez RF, Herzog H, Lee NJ. Sex-specific changes in metabolism during the transition from chow to high-fat diet feeding are abolished in response to dieting in C57BL/6J mice. *Int J Obes*. 2022;46(10):1749–1758.
10 Chang E, Varghese M, Singer K. Gender and sex differences in adipose tissue. *Curr Diab Rep*. 2018;18(9):69.
11 Ornoy A, Giron S, Aner R, Goldstein M, Boyan BD, Schwartz Z. Gender dependent effects of testosterone and 17 beta-estradiol on bone growth and modelling in young mice. *Bone Miner*. 1994;24(1):43–58.
12 Davis KE, M DN, Sun K, et al. The sexually dimorphic role of adipose and adipocyte estrogen receptors in modulating adipose tissue expansion, inflammation, and fibrosis. *Mol Metab*. 2013;2(3):227–242.
13 Heine PA, Taylor JA, Iwamoto GA, Lubahn DB, Cooke PS. Increased adipose tissue in male and female estrogen receptor-alpha knockout mice. *Proc Natl Acad Sci USA*. 2000;97(23):12729–12734.
14 Journe F, Dumon JC, Kheddoumi N, et al. Extracellular calcium downregulates estrogen receptor alpha and increases its transcriptional activity through calcium-sensing receptor in breast cancer cells. *Bone*. 2004;35(2):479–488.
15 Garrett JE, Capuano IV, Hammerland LG, et al. Molecular cloning and functional expression of human parathyroid calcium receptor cDNAs. *J Biol Chem*. 1995;270(21):12919–12925.
16 Brown EM, MacLeod RJ. Extracellular calcium sensing and extracellular calcium signaling. *Physiol Rev*. 2001;81(1):239–297.
17 Hofer AM, Brown EM. Extracellular calcium sensing and signalling. *Nat Rev Mol Cell Biol*. 2003;4(7):530–538.
18 Nemeth EF, Scarpa A. Are changes in intracellular free calcium necessary for regulating secretion in parathyroid cells? *Ann N Y Acad Sci*. 1987;493:542–551.
19 Cheng Z, Liang N, Chen TH, et al. Sex and age modify biochemical and skeletal manifestations of chronic hyperparathyroidism by altering target organ responses to Ca2+ and parathyroid hormone in mice. *J Bone Miner Res*. 2013;28(5):1087–1100.
20 Cifuentes M, Albala C, Rojas C. Calcium-sensing receptor expression in human adipocytes. *Endocrinology*. 2005;146(5):2176–2179.
21 Rocha G, Villalobos E, Fuentes C, et al. Preadipocyte proliferation is elevated by calcium sensing receptor activation. *Mol Cell Endocrinol*. 2015;412:251–256.
22 He YH, He Y, Liao XL, et al. The calcium-sensing receptor promotes adipocyte differentiation and adipogenesis through PPARγ pathway. *Mol Cell Biochem*. 2012;361(1–2):321–328.
23 Sundararaman SS, Peters LJF, Jansen Y, et al. Adipocyte calcium sensing receptor is not involved in visceral adipose tissue inflammation or atherosclerosis development in hyperlipidemic Apoe(-/-) mice. *Sci Rep*. 2021;11(1):10409.
24 Goettsch C, Hutcheson JD, Hagita S, et al. A single injection of gain-of-function mutant PCSK9 adeno-associated virus vector induces cardiovascular calcification in mice with no genetic modification. *Atherosclerosis*. 2016;251:109–118.
25 Wu T, Hu E, Xu S, et al. clusterProfiler 4.0: a universal enrichment tool for interpreting omics data. *Innovation*. 2021;2(3):100141.
26 Yu G, Wang LG, Yan GR, He QY. DOSE: an R/Bioconductor package for disease ontology semantic and enrichment analysis. *Bioinformatics*. 2015;31(4):608–609.

27 Yu G, He QY. ReactomePA: an R/Bioconductor package for reactome pathway analysis and visualization. *Mol Biosyst*. 2016;12(2):477–479.
28 Pulit SL, Stoneman C, Morris AP, et al. Meta-analysis of genome-wide association studies for body fat distribution in 694 649 individuals of European ancestry. *Hum Mol Genet*. 2019;28(1):166–174.
29 Aragam KG, Jiang T, Goel A, et al. Discovery and systematic characterization of risk variants and genes for coronary artery disease in over a million participants. *Nat Genet*. 2022;54(12):1803–1815.
30 Kheradpour P, Kellis M. Systematic discovery and characterization of regulatory motifs in ENCODE TF binding experiments. *Nucleic Acids Res*. 2014;42(5):2976–2987.
31 Vezzoli G, Terranegra A, Aloia A, et al. Decreased transcriptional activity of calcium-sensing receptor gene promoter 1 is associated with calcium nephrolithiasis. *J Clin Endocrinol Metab*. 2013;98(9):3839–3847.
32 Kawai T, Autieri MV, Scalia R. Adipose tissue inflammation and metabolic dysfunction in obesity. *Am J Physiol Cell Physiol*. 2021;320(3):C375–C391.
33 Fabbrini E, Sullivan S, Klein S. Obesity and nonalcoholic fatty liver disease: biochemical, metabolic, and clinical implications. *Hepatology*. 2010;51(2):679–689.
34 Newell-Fugate AE. The role of sex steroids in white adipose tissue adipocyte function. *Reproduction*. 2017;153(4):R133–R149.
35 Liu Q, Li R, Chen G, et al. Inhibitory effect of 17 beta-estradiol on triglyceride synthesis in skeletal muscle cells is dependent on ESR1 and not ESR2. *Mol Med Rep*. 2019;19(6):5087–5096.
36 Alexopoulos N, Katritsis D, Raggi P. Visceral adipose tissue as a source of inflammation and promoter of atherosclerosis. *Atherosclerosis*. 2014;233(1):104–112.
37 Berg AH, Scherer PE. Adipose tissue, inflammation, and cardiovascular disease. *Circ Res*. 2005;96(9):939–949.
38 Ren X, Fu X, Zhang X, et al. Testosterone regulates 3T3-L1 preadipocyte differentiation and epididymal fat accumulation in mice through modulating macrophage polarization. *Biochem Pharmacol*. 2017;140:73–88.
39 Cifuentes M, Rojas CV. Antilipolytic effect of calcium-sensing receptor in human adipocytes. *Mol Cell Biochem*. 2008;319(1–2):17–21.
40 D'Espessailles A, Mora YA, Fuentes C, Cifuentes M. Calcium-sensing receptor activates the NLRP3 inflammasome in LS14 preadipocytes mediated by ERK1/2 signaling. *J Cell Physiol*. 2018;233(8):6232–6240.
41 Mattar P, Sanhueza S, Yuri G, et al. Calcium-sensing receptor in adipose tissue: possible association with obesity-related elevated autophagy. *Int J Mol Sci*. 2020;21(20):7617.
42 Nishimura S, Manabe I, Nagasaki M, et al. CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. *Nat Med*. 2009;15(8):914–920.
43 Wang Q, Wang Y, Xu D. The roles of T cells in obese adipose tissue inflammation. *Adipocyte*. 2021;10(1):435–445.
44 Kaikaew K, Steenbergen J, van Dijk TH, Grefhorst A, Visser JA. Sex difference in corticosterone-induced insulin resistance in mice. *Endocrinology*. 2019;160(10):2367–2387.
45 Cristancho AG, Lazar MA. Forming functional fat: a growing understanding of adipocyte differentiation. *Nat Rev Mol Cell Biol*. 2011;12(11):722–734.
46 Hu E, Tontonoz P, Spiegelman BM. Transdifferentiation of myoblasts by the adipogenic transcription factors PPAR gamma and C/EBP alpha. *Proc Natl Acad Sci U S A*. 1995;92(21):9856–9860.
47 Sun C, Mao S, Chen S, Zhang W, Liu C. PPARs-orchestrated metabolic homeostasis in the adipose tissue. *Int J Mol Sci*. 2021;22(16):8974.
48 He Y, Zhang H, Teng J, Huang L, Li Y, Sun C. Involvement of calcium-sensing receptor in inhibition of lipolysis through intracellular cAMP and calcium pathways in human adipocytes. *Biochem Biophys Res Commun*. 2011;404(1):393–399.
49 Sylvers-Davie KL, Davies BSJ. Regulation of lipoprotein metabolism by ANGPTL3, ANGPTL4, and ANGPTL8. *Am J Physiol Endocrinol Metab*. 2021;321(4):E493–e508.
50 Pan C, Lei Z, Wang S, et al. Genome-wide identification of cyclin-dependent kinase (CDK) genes affecting adipocyte differentiation in cattle. *BMC Genom*. 2021;22(1):532.
51 Canaff L, Hendy GN. Calcium-sensing receptor gene transcription is up-regulated by the proinflammatory cytokine, interleukin-1 beta. Role of the NF-kappaB PATHWAY and kappaB elements. *J Biol Chem*. 2005;280(14):14177–14188.
52 Kern PA, Saghizadeh M, Ong JM, Bosch RJ, Deem R, Simsolo RB. The expression of tumor necrosis factor in human adipose tissue. Regulation by obesity, weight loss, and relationship to lipoprotein lipase. *J Clin Investig*. 1995;95(5):2111–2119.
53 Tokgozoglu L, Libby P. The dawn of a new era of targeted lipid-lowering therapies. *Eur Heart J*. 2022;43(34):3198–3208.
54 Robinet P, Milewicz DM, Cassis LA, Leeper NJ, Lu HS, Smith JD. Consideration of sex differences in design and reporting of experimental arterial pathology studies-statement from ATVB council. *Arterioscler Thromb Vasc Biol*. 2018;38(2):292–303.
55 Roeters van Lennep JE, Tokgozoglu LS, Badimon L, et al. Women, lipids, and atherosclerotic cardiovascular disease: a call to action from the European Atherosclerosis Society. *Eur Heart J*. 2023;44(39):4157–4173.