

Original Article

Longitudinal Profiling Reveals Divergent Kidney and Brain Immune Responses during Metabolic Syndrome

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ABSTRACT: Chronic kidney disease (CKD) and cerebral small vessel disease (cSVD) share major cardiometabolic risk factors, including hypertension, diabetes, and obesity. The immune system may play a key role in kidney-brain interactions and disease development. However, whether circulating inflammatory mediators reflect similar immune responses in the kidney and brain remains unclear. We therefore characterized circulating, renal and cortical cerebral responses in a rat model of metabolic syndrome. Lean and Obese ZSF1 rats were studied at 8–9, 22–23, and 34–35 weeks of age. Systemic inflammation was assessed using multiplex plasma cytokine/chemokine analysis and flow cytometry of circulating immune cells. Kidney injury was evaluated by histopathology and expression of injury markers. Renal and cerebral blood flow were measured by Laser Speckle Contrast Imaging. Renal macrophage accumulation and cortical cerebral immune cell readouts were assessed by immunohistochemistry. Progression of metabolic syndrome in Obese ZSF1 rats was associated with elevated circulating inflammatory mediators, including IP-10, MCP-1, and RANTES, together with increased classical monocytes from 8–9 weeks and non-classical monocytes from 22–23 weeks onward. Kidney pathology was further characterized by progressive macrophage accumulation and reduced renal blood flow. In contrast, cortical cerebral analyses showed no evidence of macrophage infiltration, while CD68 positivity was reduced in Iba1+ microglia in Obese rats at 34–35 weeks. Despite marked systemic inflammation and renal immune activation, the cerebral cortex displayed a distinct immune response with no macrophage infiltration and altered microglial activity. Rather than indicating a uniform inflammatory response across organs, these descriptive findings provide insight into kidney–brain immune divergence during metabolic syndrome.

Keywords: diabetic nephropathy, vascular cognitive impairment, comorbidities, immune cells, macrophages, microglia

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INTRODUCTION

Chronic kidney disease (CKD) is a major health burden that affects >10% of the general population worldwide, which corresponds to >800 million people [1]. CKD may be part of a multisystem small vessel disorder leading to reduced perfusion of various organ systems [2]. The organ systems affected by small vessel disease (SVD) involve those that receive substantial portions of cardiac output such as the kidney and the brain [2]. In the brain, SVD may lead to vascular cognitive impairment (VCI). Previous studies have shown that CKD is associated with MRI markers of cerebral small vessel disease (cSVD) such as white matter hyperintensities (WMH), lacunes, cerebral microbleeds (CMBs), and enlarged perivascular spaces (ePVS) [3]. CKD and cSVD share many of the same risk factors, including hypertension, obesity, and diabetes [4]. As the prevalence of these risk factors continues to rise, the incidence of CKD and cSVD will also increase [5, 6]. Despite the increase in CKD and cSVD burden, the exact pathophysiological mechanisms connecting CKD and cSVD are still poorly understood. Therapeutic options are therefore limited, with risk factor management remaining the sole solution for CKD and cSVD prevention and treatment [7, 8].

A key feature of both CKD and cSVD is the presence of persistent, low-grade inflammation, characterized by elevated levels of circulating cytokines and chemokines [9]. Due to the circulating nature of many inflammatory mediators, it is hypothesized that the immune system could have crucial roles in organ interactions and disease development [9]. In CKD, the dysregulation of immune responses includes a complex interplay between various immune cell types, including resident and infiltrating immune cells, cytokines, chemokines, and complement factors [10]. These immune factors can trigger and sustain kidney inflammation, causing renal tissue injury and progressive fibrosis [10]. While the inflammatory processes have been well described in CKD, the role of immune responses in cSVD remains poorly understood. Moreover, key players in the cerebro-renal connection remain largely unknown, particularly when risk factors are combined. While systemic inflammation is a recognized contributor to both diseases, the extent to which local inflammatory responses within the kidney and brain mirror or diverge from systemic patterns remains to be elucidated.

In this study, we aimed to longitudinally characterize circulating and local inflammatory mediators and the associated renal and cerebral phenotypes in a rat model of metabolic syndrome. We studied the ZSF1 rat model, a well-established model for CKD [11], in which we have recently reported neurovascular dysfunction and cognitive impairment in Obese rats [12]. We investigated

the effect of combined risk factors on circulating and local inflammatory mediators (immune cells and cytokines) at three different timepoints (8–9w, 22–23w, 34–35w) associated with disease progression.

METHODS

Animals and study design

All experiments were approved by the Ethical Committees for Animal Experiments of Maastricht University (AVD10700202010326) and were performed according to institutional guidelines. Experiments were performed with male Zucker fatty and spontaneously hypertensive (ZSF1) rats (Charles River Inc.), which is a cross between the Spontaneous Hypertensive Heart Failure (SHHF) rat and the Zucker Diabetic Fat (ZDF) rat. Obese ZSF1 rats develop hypertension, obesity, and type 2 diabetes, whereas Lean animals present with only hypertension (Schauer 2020). Lean and Obese rats were studied at an early timepoint before the development of kidney failure (8–9 weeks), an intermediate timepoint (22–23 weeks) when Obese rats present with kidney dysfunction, and a late timepoint of prolonged exposure to risk factors and the presence of cognitive impairment (34–35 weeks) [12, 13]. These ages refer to the terminal endpoints for each animal batch. Cerebral blood flow was assessed before terminal procedures at 6–7, 20–21, and 32–33 weeks, whereas renal blood flow was assessed during the terminal procedure and is therefore reported at 8–9, 22–23, and 34–35 weeks. Animals were socially housed under stable conditions (12/12-h inverted day/night cycle, 22±1°C, relative humidity level of 55%, soft-playing radio) and had access to food (v1124-703, SSNIFF Spezialdiäten GmbH) and water ad libitum. Body weights were measured weekly. Systolic and diastolic blood pressures were measured in awake rats during their active phase with the CODA tail-cuff system (Kent Scientific corporation) using volume pressure recording. Prior to the start of the experiment, animals were habituated to a restrainer for 15 minutes in a warming cabinet (28°C) for three consecutive days. The development of hypertension, diabetes, and obesity in ZSF1 rats (n=14/group) was assessed as described in Lambrichts *et al.* [12]. The progression of cognitive impairment was also previously reported in this model, but behavioral/cognitive outcomes were assessed in a separate cohort and not in the same animals used for the present immune and histological analyses.

Laser Speckle Contrast Imaging

At 6–7w, 20–21w, and 32–33w, thinned skull window surgeries were performed to measure cerebral blood flow

using laser speckle contrast imaging (LSCI) as previously described in Lambrichts *et al.* [12]. One hour prior to sacrifice, rats were injected with 0.05 mg/kg buprenorphine (s.c., Bupaq, Fendigo, Pharma AG). The superficial perfusion of the renal cortex from Lean and Obese rats (n=10/group) was measured using LSCI at 8-9w, 22-23w and 34-35w. First, animals were anaesthetized by i.p. injection with a mixture of 75 mg/kg ketamine and 0.5 mg/kg medetomidine. Next, the animal was moved to the heating table and the left kidney was exposed, freed from surrounding fat, and kept moisturized with warm saline solution. Kidney cortical perfusion at baseline was then measured using LSCI after 5 min of flux stabilization (PeriCam PSI HR, Perimed, resolution = 10 $\mu\text{m}/\text{pixel}$, image size = 5 x 5 mm; frame averaging = 10; frame rate = 44 images/second). Analysis was accomplished using PIMSoft 1.11 software (Perimed).

Sample collection

During terminal procedure, anaesthesia was switched to 4% isoflurane and blood was withdrawn from the *vena cava*, followed by terminal pentobarbital injection (i.v., 100 mg/kg, Nembutal, Ceva) and cardiac perfusion with ice-cold PBS supplemented with 1% EDTA (pH 8.0, ThermoFisher). The kidney, brain, plasma and urine samples were prepared for further analyses and either snap frozen in liquid nitrogen or fixed with 4% paraformaldehyde overnight (PFA, Sigma-Aldrich). In addition, brains and kidneys were weighed on a precision scale (Mettler Toledo, Switzerland), whereas tibia length was measured using a vein caliper. Plasma and urine molecular analysis was performed as described in Lambrichts *et al.* [12].

Oxidative stress marker in urine

Urinary oxidative 8-Oxo-dG was analyzed by ultra-performance liquid chromatography (Acquity UPLC, Waters, Milford, USA) and detected in multiple reaction monitoring electrospray ionization positive mode (MRM-ESI) using a Xevo TQ-XS (Waters, Milford, USA). In short, urinary 8-Oxo-dG (100 μL urine) was extracted using an SPE cleanup with a 96-well Oasis HLB $\mu\text{Elution}$ plate (Waters, Milford, USA) and subsequently dried under a gentle stream of nitrogen at 40°C. The residue was dissolved in 200 μL acetonitrile:water (2:98, v/v) and injected on a reversed-phase UPLC column (Waters, Acquity UPLC HSS T3, 100x2.1 mm) using an ion-pair/acetonitrile gradient. Injection volume was 10 μL (full loop injection) at a column temperature of 45°C. MRM-ESI was performed at a capillary voltage of 0.5 kV, a cone voltage of 5-20 V and a desolvation temperature of 600°C. Quantitation of 8-Oxo-dG was done by calculating

the peak area ratio to the corresponding stable isotope, which was used as internal standard. The calibration was linear over physiological concentration ranges ($r^2 > 0.99$) in both water and urine matrix. Inter- and intra-assay variation as determined by replicate analysis of a urine sample on 5 different days and 5 replicate analysis on one day were ~6% (CV%).

Peripheral blood mononuclear cell isolation and flow cytometry

For identification of circulating immune cells in Lean and Obese ZSF1 rats (n=10/group), blood was collected into EDTA-lined tubes followed by peripheral blood mononuclear cell (PBMC) isolation via SepMate™ density gradient centrifugation (STEMCELL Technologies Inc.). Blood was diluted with an equal volume of D-PBS (at RT), layered on top of Lymphoprep™ (STEMCELL Technologies Inc., at RT), and centrifuged (1200g, 10 min, 21°C). PBMCs were collected, counted and assessed for viability. For cryopreservation of PBMCs, isolated cells were resuspended in ice-cold freezing medium (RPMI-1640 medium containing 40% FBS and 20% DMSO) and placed in a Nalgene Cryo 1 °C freezing container with isopropanol and frozen overnight at -80 °C, before being transferred to liquid nitrogen. At the time of analysis, PBMCs were thawed in a 37° C water bath and assessed for viability.

After live/dead staining using Fixable Viability Dye eFluor 780 (1:1000, 65-0865-14, ThermoFisher Scientific), the following antibodies were used: anti-rat CD45-Alexa fluor 700 (1:40, 202218, BioLegend), anti-rat CD3-VioGreen (1:100, 130-119-125, Miltenyi Biotec), anti-rat CD4-V450 (1:100, 561579, BD Biosciences), anti-rat CD8-PerCP-eFluor 710 (1:100, 46-0084-82, ThermoFisher), anti-rat CD43-PE (1:100, 202812, BioLegend), anti-rat His48-FITC (1:100, 11-0570-82, ThermoFisher), anti-rat CD161-APC (1:100, 205606, BioLegend), CD45R-Pe-Cy7 (1:100, 25-0460-82, BioLegend). Flow cytometry was performed using Cytex Aurora (Cytex Biosciences, Inc.) and analysis was performed using FlowJo 10.8.0 (BD Biosciences).

Cytokine plasma panel

Twenty-seven cytokines, chemokines, and growth factors were simultaneously measured in plasma samples from Lean and Obese rats (n=10/group) using Eve Technologies' Rat Cytokine 27-Plex Discovery Assay® (MilliporeSigma) according to the manufacturer's protocol. The multiplexing analysis was performed using the Luminex™ 200 system by Eve Technologies Corp. (Calgary, Alberta). The 27-plex consisted of EGF,

Eotaxin, Fractalkine, G-CSF, GM-CSF, GRO/KC, IFN γ , IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12(p70), IL-13, IL-17A, IL-18, IP-10, Leptin, LIX, MCP-1, MIP-1 α , MIP-2, RANTES, TNF α , and VEGF. Assay sensitivities of these markers range from 0.3 – 30.7 pg/mL. Individual analyte sensitivity values are available in the MilliporeSigma MILLIPLEX® MAP protocol. A heatmap was created with log2 transformed and scaled data using the pheatmap package in R. Hierarchical clustering was performed using correlation distance and complete linkage clustering. PCA was performed separately for each timepoint (8-9w, 22-23w, 34-35w) using the prcomp function with unit variance scaling. PCA loadings plots were generated to visualize the contribution of individual cytokines to PC1 and PC2. Scree plots were created using the factoextra package to assess the variance explained by each principal component. Analyses were conducted in R using the ggplot2, tidyr, factoextra, and dplyr packages.

RT-PCR

Following transverse sectioning, the upper half of the right kidney was used for RNA extraction. Total RNA from kidney tissue was obtained using TriPure™ Isolation Reagent (Sigma-Aldrich). The RNA concentration after extraction was around 1145 ng/ μ L per sample and 2 μ g was reversed transcribed to cDNA with NZY First-Strand cDNA Synthesis Kit (Nzytech). Reverse transcription-polymerase chain reaction (RT-PCR) was performed in a 7500 Fast ABI System (Invitrogen Life Technologies Inc., Carlsbad, CA, USA) in duplicate to ensure the reliability of RT-PCR values, using specific qPCRs probes and assays (Table S1). Data were analyzed using the average of the duplicate values. β -actin (Actb) showed minimal variation across experimental conditions (mean $\pm$ SD Ct = 20,4 $\pm$ 0,8), with a maximal Δ Ct < 1 across all groups and timepoints and was used as a housekeeping gene. PCR cycling conditions were: initial denaturation at 95°C for 30 s, 40 cycles at 95°C for 5 s and 60°C for 30 s, followed by a melting curve. To analyze the changes in gene expression, the 2^{− $\Delta\Delta$ Ct} method was employed. Data are expressed as fold change over control.

NADPH oxidase activity assay

The O₂^{•−} production generated by NADPH oxidase was determined by a chemiluminescence assay. Frozen rat kidney samples were homogenized in ice-cold phosphate buffer (50mmol/L KH₂PO₄, 1mmol/L EGTA, 150mmol/L sucrose, pH 7.4). The reaction was performed in kidney homogenates starting by the addition of a lucigenin (5 μ mol/L) and NADPH (100 μ mol/L; Sigma-Aldrich)

mixture to the protein sample in a final volume of 250 μ L. Chemiluminescence was determined every 2.4 s for 3 min in a microtiter plate luminometer (Enspire Perkin Elmer). Basal activity in the absence of NADPH was subtracted from each reading and normalized to protein concentration. Protein content of kidney samples was detected by Pierce™ BCA Protein Assay Kit.

Histological assessment of kidney and brain injury

For histopathological examination, fixed kidney samples (n=10/group) were dehydrated, paraffin embedded and cut into 4 μ m sections using a microtome. Brains (n=10/group) were sliced into free-floating sections of 30 μ m using a vibratome (Leica VT1200, Leica Microsystems). To assess kidney injury and renal and cerebral immune cell infiltration, sections were stained with Hematoxylin and Eosin (H&E). The sections were investigated by a pathologist, and pictures were taken using a brightfield slide scanner (Olympus BX61VS) at 10 x magnification.

Immunohistochemistry

To quantify CD68+ macrophages in kidneys of Lean and Obese rats, PFA fixed sections were deparaffinized, hydrated, and blocked with 30% H₂O₂ for 10 min each at RT. Next, antigen retrieval was performed with citrate buffer (0.01M) for 10 min at 95°C. Primary antibody includes mouse anti-CD68 (1:500, MCA341R, BIO-RAD), which were diluted in 0.1% Tween in PBS and incubated overnight at 4°C. Following washing, sections were incubated with BrightVision goat anti-HRP for 30 min at RT. Next, sections were incubated with DAB, counterstained with haematoxylin and mounted with Entellan mounting medium. For every animal, 10 field of views from two different sections were analysed. Pictures were taken across all renal compartments using an inverted microscope (Axio Vert. A1, Zeiss) at 20 x magnification. Using ImageJ software (ImageJ, NIH, Bethesda, MD, USA, RRID:SCR_001935), intensity thresholding was applied to the images by setting a lower and upper threshold which were then converted to binary format. Finally, macrophage area was measured. Data analysis was carried out with the averaged CD68+ areas of the images of each rat.

To quantify the macrophages and microglia density in the brain, free-floating sections were blocked using 3% donkey serum in PBS containing 0.5% Triton-X-100 overnight at 4°C. Primary antibodies included mouse anti-rat CD68 (1:500, MCA341R, BIO-RAD), rabbit anti-rat Iba1 (1:500, 019-19741, WAKO) and goat anti-mouse CD206 (1:500; R&D AF2535), which were diluted in 0.3% donkey serum in PBS-T and incubated overnight at

4°C. Following washing, secondary antibody incubation was done for 2h at RT. The following secondary antibodies were used: donkey-anti mouse Alexa fluor 647 (1:500, A-31571, Invitrogen), donkey anti-rabbit Alexa fluor 488 (1:500, A-21206, Invitrogen) and donkey anti-goat Alexa Fluor 594 (1:400, A-11058, Invitrogen). Vessel labelling was achieved using either Tomato-lectin Texas red (1:400, TL-1176-1, Vector Laboratories, Inc.) or Tomato-lectin Dylight488 (1:500, TL-1174-1, Vector Laboratories Inc.) overnight at 4°C. Counterstaining with DAPI (NucBlue, R37606, Invitrogen) was performed for 10 min. Images were acquired from the cerebral cortex (ImageXpress Pico, Molecular Devices, San Jose, USA) to ensure consistency with the vascular readouts obtained in the original study, including superficial cortical blood flow and transcriptomic profiling of isolated cortical microvessels. In addition, cortical regions showed clear IgG extravasation in our dataset, supporting their relevance for assessing microglial responses in areas affected by vascular leakage. The number of CD68+ Iba1+ cells was assessed in Arivis Vision 4D (version 3.6.2, Zeiss, Rostock, Germany). Two sections per animal with three regions of interest/section were analyzed. In short, a machine learning segmentation was performed to identify background (tissue negative for CD68 and Iba1), macrophages (CD68-positive signal), and microglia (Iba1-positive signal). The obtained objects were filtered to remove artificial background signal. Iba1+ objects with a volume >600µm³ and a volume-to-voxel count ratio <9000 were excluded from further analysis. Using compartmentalization analysis, the CD68+ Iba1+ cells

were identified and the total number of Iba1+ cells, CD68+ cells, and CD68+ Iba1+ cells were exported for analysis. For the quantification of perivascular macrophages (PVMs), images were obtained utilising confocal microscopy (Leica DMI 4000B, Leica Microsystems, Wetzlar, Germany) at a 40x magnification (ACS APO 40.0×1.15 OIL). Per brain section, two FOVs of 275 µm x 275 µm from the cortex were obtained containing at least one large vessel (diameter > 10 µm) from 8 µm z-stacks. Maximal intensity projections were created from the obtained images for further analysis. Blinded images were analysed by two blinded independent observers using Image J2 version 2.16.0/1.54p. The length of the largest lectin+ vessel with a diameter > 10 µm was measured, the corresponding PVM count was determined. PVMs were defined as CD206+ cells with elongated, round or slightly ramified morphology localised around large vessels. The PVM count was afterwards corrected for vessel length.

Statistical analysis

Data are represented as mean ± standard error of the mean (SEM). All statistical analyses were performed with GraphPad Prism 10.0.1 software (San Diego, USA). Normality distribution was tested with the Shapiro-Wilk test. Two-way analysis of variance (ANOVA) was used to compare Lean and Obese groups over time, followed by Tukey's post hoc testing. Differences with P values < 0.05 were considered significant (**P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001).

Table 1. Cardio-metabolic changes in Lean and Obese ZSF1 rats at 8-9 weeks (8-9w), 22-23 weeks (22-23w) and 34-35 weeks (34-35w).

	Lean			Obese		
	8-9w	22-23w	34-35w	8-9w	22-23w	34-35w
Body weight (g)	263.8 ± 3.19	410.6 ± 5.37	467.4 ± 8.33	374.4 ± 3.36****	584.2 ± 20.0****	733.2 ± 8.76****
Brain/Tibia (mg/mm)	54 ± 0.428	49 ± 0.365	48 ± 0.447	51 ± 1.010***	46 ± 0.512***	47 ± 0.367
Kidney/Tibia (mg/mm)	43 ± 0.947	46 ± 1.147	45 ± 0.767	57 ± 1.635****	68 ± 2.240****	67 ± 1.660****
Mean systolic blood pressure (mmHg)	146.7 ± 3.75	152.2 ± 2.47	152.4 ± 2.06	144.2 ± 2.91	165.7 ± 2.45****	159.8 ± 3.63
Fasting plasma glucose (mg/dl)	89.4 ± 2.17	90.5 ± 1.90	89.6 ± 3.16	108.4 ± 5.09**	110.6 ± 3.85**	115.8 ± 4.84***
Plasma triglycerides (mmol/L)	0.364 ± 0.05	0.486 ± 0.04	0.450 ± 0.05	3.208 ± 0.89	24.39 ± 3.35****	14.48 ± 1.76****
Plasma urea (mmol/L)	6.55 ± 0.66	6.79 ± 0.23	7.89 ± 0.19	9.29 ± 1.45**	8.80 ± 0.39**	8.59 ± 0.26
Urine glucose (mmol/L)	3.20 ± 1.30	2.79 ± 0.77	4.42 ± 1.08	167.0 ± 39.6****	119.8 ± 16.0****	64.1 ± 9.16*

Data are mean ± SEM. 2-way ANOVA with Tukey's multiple comparison test. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001 compared to age-matched Lean rats. n=9-10/group.

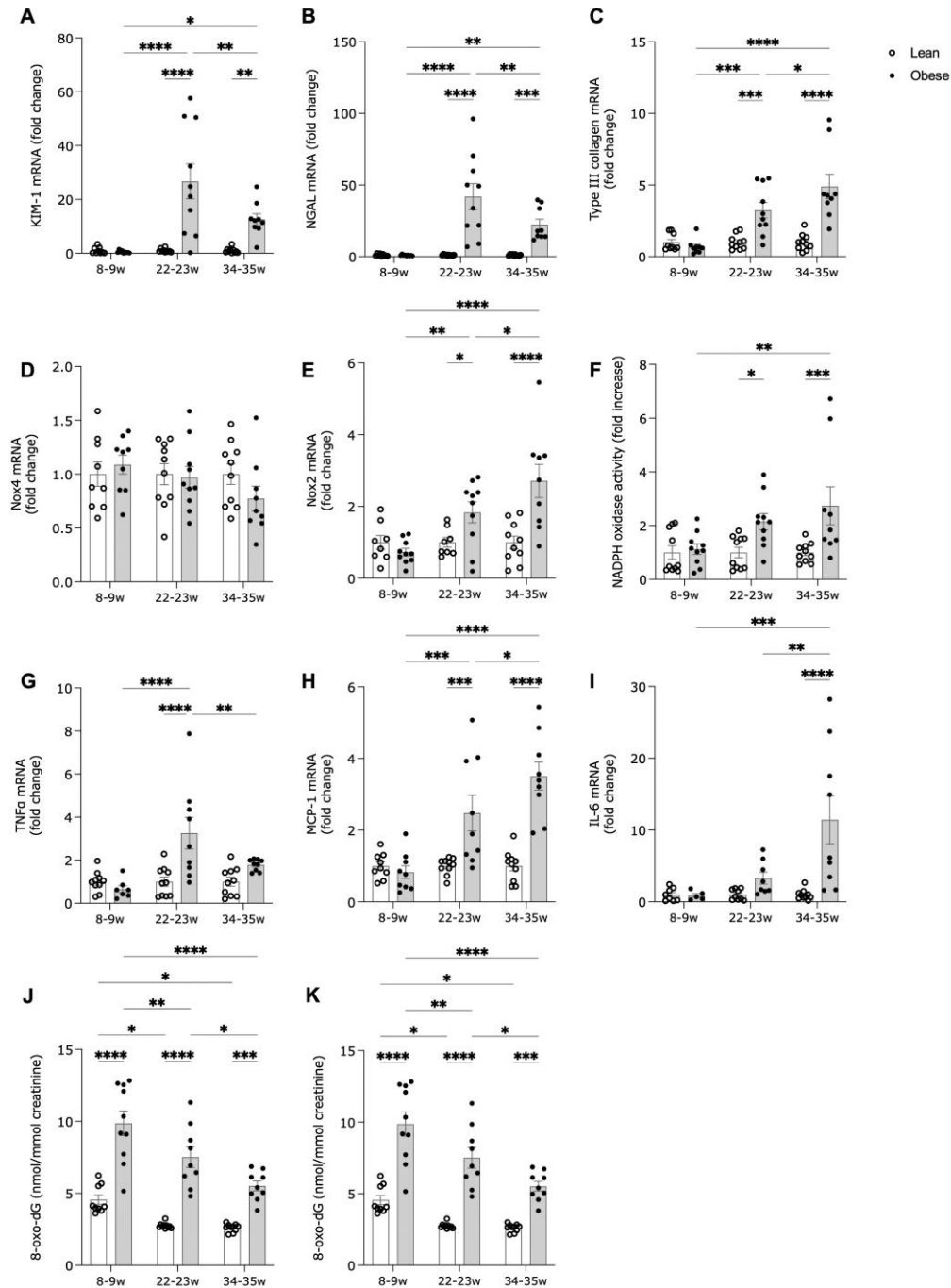


Figure 1. Obese ZSF1 rats develop progressive renal injury associated with oxidative stress and inflammatory activation. Gene expression levels in Obese and Lean rats at 8-9 weeks (8-9w), 22-23 weeks (22-23w) and 34-35 weeks (34-35w) of (A) KIM-1 (2-way ANOVA: $p_{\text{page}}=0.0002$, $p_{\text{group}}<0.0001$, $p_{\text{page} \times \text{group}}=0.0002$), (B) NGAL (2-way ANOVA: $p_{\text{page}}<0.0001$, $p_{\text{group}}<0.0001$, $p_{\text{page} \times \text{group}}<0.0001$), (C) type III collagen (2-way ANOVA: $p_{\text{page}}<0.0001$, $p_{\text{group}}<0.0001$, $p_{\text{page} \times \text{group}}<0.0001$), (D) Nox4 (2-way ANOVA: $p_{\text{page}}=0.3117$, $p_{\text{group}}=0.5043$, $p_{\text{page} \times \text{group}}=0.3117$), (E) Nox2 (2-way ANOVA: $p_{\text{page}}=0.0016$, $p_{\text{group}}=0.0008$, $p_{\text{page} \times \text{group}}=0.0016$), (F) NADPH oxidase activity (2-way ANOVA: $p_{\text{page}}=0.0597$, $p_{\text{group}}=0.0005$, $p_{\text{page} \times \text{group}}=0.0597$), (G) TNFα (2-way ANOVA: $p_{\text{page}}=0.0016$, $p_{\text{group}}=0.0008$, $p_{\text{page} \times \text{group}}=0.0016$), (H) MCP-1 (2-way ANOVA: $p_{\text{page}}=0.0597$, $p_{\text{group}}=0.0005$, $p_{\text{page} \times \text{group}}=0.0597$), (I) IL-6 (2-way ANOVA: $p_{\text{page}}=0.0597$, $p_{\text{group}}=0.0005$, $p_{\text{page} \times \text{group}}=0.0597$), (J) ISG15 (2-way ANOVA: $p_{\text{page}}<0.0001$, $p_{\text{group}}<0.0001$, $p_{\text{page} \times \text{group}}<0.0001$) (K) Urine levels of 8-Oxo-2'-deoxyguanosine (8-Oxo-dG). Data are presented as mean ± SEM. Tukey's multiple comparisons test: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. n=8-10 rats/group.

RESULTS

Metabolic syndrome and kidney damage in Obese ZSF1 rats

Lean and obese ZSF1 rats were studied from 8 weeks to 35 weeks of age, during which the development of diabetes and hypertension was assessed. Obese ZSF1 rats had a higher body weight than Lean rats at all timepoints ($p < 0.0001$) (Table 1). When normalized to tibia lengths, brain weight was decreased in obese ZSF1 rats at 8-9w and 22-23w (p_{8-9w} and $p_{22-23w} < 0.001$). At all three timepoints, kidney-to-tibia weight was increased in obese rats compared to Lean ($p < 0.0001$). Systolic blood pressure was elevated in both Lean and Obese rats (single t-test: $p < 0.0001$ vs $t = 120$ mmHg). Moreover, obese ZSF1 rats showed a higher systolic blood pressure compared to Lean rats at 20w ($p < 0.01$). Obese rats had increased fasting plasma glucose (p_{8-9w} and $p_{22-23w} < 0.01$, $p_{34-35w} < 0.0001$) as well as urine glucose (p_{8-9w} and $p_{22-23w} < 0.0001$, $p_{34-35w} < 0.05$) at all timepoints. In addition, plasma triglyceride levels were increased in Obese vs Lean at 22-23w ($p < 0.0001$) and 34-35w ($p < 0.0001$). Plasma urea was increased in Obese vs Lean at 8-9w and 22-23w (p_{8-9w} and $p_{22-23w} < 0.01$).

To further assess the impact of metabolic syndrome on kidney phenotype, we investigated the differential expression of kidney injury markers Kidney Injury Molecule KIM-1 and Neutrophil gelatinase-associated lipocalin (NGAL), and Type III Collagen in kidneys of Lean and Obese rats at all timepoints using RT-PCR (**Fig. 1**). Increased gene expression values of KIM1 ($p_{22-23w} < 0.0001$, $p_{34-35w} < 0.01$), NGAL ($p_{22-23w} < 0.0001$, $p_{34-35w} < 0.001$), and Type III collagen ($p_{22-23w} < 0.001$, $p_{34-35w} < 0.0001$) were shown in Obese rats at 22-23w and 34-35w. Moreover, we assessed oxidative stress markers in kidney and urine of ZSF1 rats. RT-PCR revealed no significant difference in NADPH Oxidase (Nox)4 expression between Lean and Obese rats over time. On the other hand, Nox2 expression was increased in Obese rats at 22-23w and 34-35w ($p_{22-23w} < 0.05$, $p_{34-35w} < 0.0001$). Moreover, Nox2 expression significantly increased over time in Obese rats at 34-35w vs 8-9w ($p < 0.0001$). Similarly, NADPH oxidase activity was increased in Obese vs Lean at 22-23w and 34-35w ($p_{22-23w} < 0.05$, $p_{34-35w} < 0.001$) and increased over time in Obese rats at 34-35w vs 8-9w ($p < 0.01$).

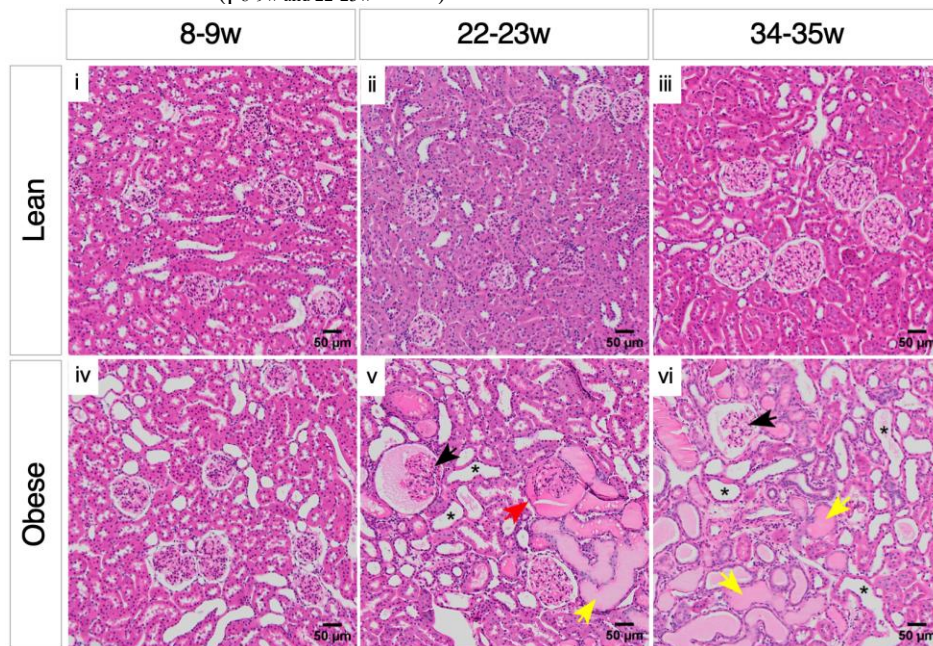


Figure 2. Obese ZSF1 rats develop progressive renal histopathological alterations. Normal histology of the kidney tissue in Lean and Obese rats at (i-iv) 8-9 weeks (8-9w), (ii-v) 22-23 weeks (22-23w), and (iii-vi) 34-35 weeks (34-35w). Tubule dilation (asterisk), glomerular degeneration (black arrow), glomerular basement membrane thickening (red arrow), protein casts (yellow arrow) in obese rats at 22-23w and 34-35w. $n = 10$ /group. Scale bar = $50\mu\text{m}$.

We next investigated inflammatory responses in the kidney. In Obese rats, an increase in TNF α and IL-6 expression was observed at 22-23w and 34-35w, respectively (p_{22-23w} and $p_{34-35w} < 0.0001$). Furthermore, an

increase in Monocyte Chemoattractant protein MCP-1 was observed in Obese rats at 22-23w and 34-35w ($p_{22-23w} < 0.001$, $p_{34-35w} < 0.0001$). MCP-1 and IL-6 expression increased in Obese rats from 8-9w to 34-35w ($p_{\text{MCP-1}}$

$p < 0.0001$, $p_{IL-6} < 0.001$). An increase in Interferon-stimulated gene 15 (ISG15) expression was observed in Obese rats at 22–23w and 34–35w ($p < 0.0001$). Furthermore, ISG15 increased in Obese rats from 8–9w to 34–35w ($p < 0.0001$). Finally, the urinary biomarker 8-hydroxy-2'-deoxyguanosine (8-Oxo-dG) was measured in lean and obese rats using UPLC/MS-MS. At all timepoints, 8-oxo-dG was increased in Obese ZSF1 rats (p_{8-9w} and $22-23w < 0.0001$, $p_{34-35w} < 0.001$). However, 8-oxo-dG decreased in both Lean and Obese rats over time at 34–35w vs 8–9w ($p_{lean} < 0.05$, $p_{obese} < 0.0001$).

Complementing previous analyses, we assessed histopathological changes in kidney sections from Lean and Obese rats (Fig. 2). No significant histological changes were found between lean and obese rats at 8–9w.

At 22–23w and 34–35w, Obese rats showed tubular dilation and atrophy, glomerular basement membrane thickening, glomerular degeneration, and protein casts.

Renal and cerebral blood flow

Baseline renal and cerebral blood flow were assessed in lean and obese ZSF1 rats at three different timepoints using LSCI. While baseline renal blood flow decreased in Obese rats at 33–34w, baseline cerebral blood flow remained unchanged between Lean and Obese rats over time (Fig. 3). Moreover, renal blood flow significantly decreased from 22–23w to 34–35w ($p < 0.01$) while baseline cerebral blood flow was slightly decreased in Obese rats at 32–33w vs 6–7w ($p = 0.0519$).

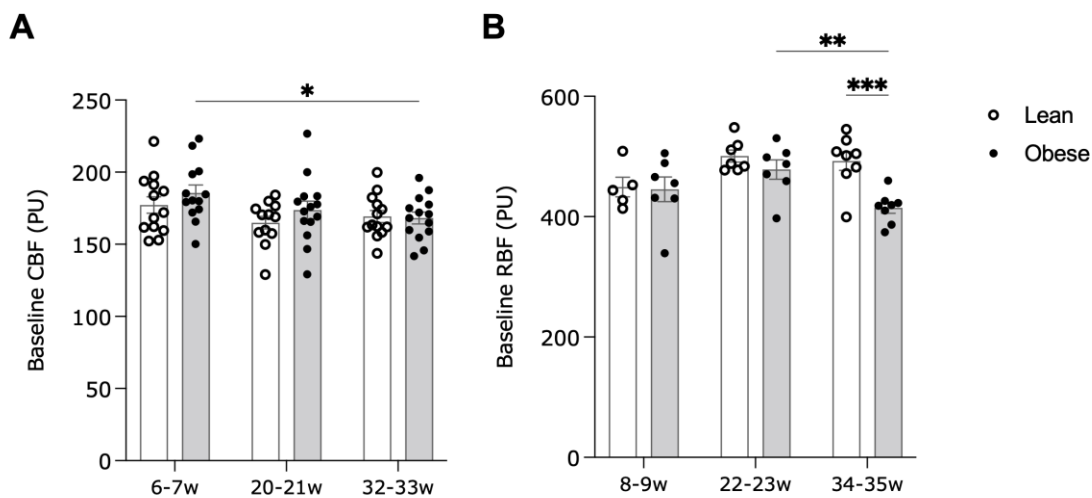


Figure 3. Obese ZSF1 rats show reduced renal blood flow with preserved resting cerebral blood flow. Baseline cerebral blood flow (CBF) and renal blood flow (RBF) of Lean and Obese ZSF1 rats were assessed at three different timepoints using LSCI. (A) Baseline CBF of lean and obese ZSF1 rats measured at 6–7 weeks (6–7w), 20–21 weeks (20–21w), and 32–33 weeks (32–33w) (2-way ANOVA: $p_{age} = 0.026$, $p_{group} = 0.203$, $p_{age \times group} = 0.552$). (B) Baseline RBF of Lean and Obese ZSF1 rats measured at 8–9 weeks (8–9w), 22–23 weeks (22–23w), and 34–35 weeks (34–35w) (2-way ANOVA: $p_{age} = 0.018$, $p_{group} = 0.008$, $p_{age \times group} = 0.046$). Data are presented as mean $\pm$ SEM. Tukey's multiple comparisons test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 5$ –14 rats/group.

Circulating immune cells and inflammatory markers

To better understand the underlying mechanisms contributing to the renal and cerebral phenotype, we investigated systemic and organ-specific inflammatory mediators. Flow cytometry was performed to investigate circulating immune cell populations over time in Lean and Obese ZSF1 rats (Fig. 4, Supplementary Fig. 1). At 34–35w, CD3⁺ T cells were decreased in obese vs lean ($p < 0.05$). At 8–9w and 34–35w, CD8⁺ Cytotoxic T cells were decreased in obese rats ($p_{8-9w} < 0.05$; $p_{34-35w} < 0.001$). At all timepoints, CD4⁺ T cells were increased in obese vs lean (p_{8-9w} and $22-23w < 0.05$, $p_{34-35w} < 0.001$). No significant difference in NK cells was found. At 8–9w, B cells were decreased in Obese rats ($p < 0.001$). Non-classical

monocytes were increased at 22–23w and 34–35w in Obese rats by 7.5% compared to Lean (p_{22-23w} and $34-35w < 0.01$). At all timepoints, classical monocytes were increased by 4–5% in Obese vs Lean ($p_{8-9w} < 0.05$, $p_{22-23w} < 0.001$, $p_{34-35w} < 0.0001$).

Alongside the identification of immune cell populations, 27 cytokines, chemokines, and growth factors were measured in the plasma of lean and obese ZSF1 rats at 8–9w, 22–23w, and 34–35w. Analytes include inflammatory cytokines [Interleukin IL-1 α , IL-1 β , IL-6, IL-12(p70), IL-18, and tumour necrosis factor (TNF)- α], chemokines [macrophage inflammatory protein MIP-1 α , MIP-2, interferon gamma-induced protein (IP)-10, monocyte chemoattractant protein MCP-1, Regulated on Activation, Normal T Expressed and Secreted

(RANTES), LIX, Fractalkine, and eotaxin], adaptive cytokines [IL-2, IL-4, IL-5, IL-13, IL-17A, and interferon(IFN)- γ], growth factors [granulocyte macrophage-colony stimulating factor (GM-CSF), granulocyte-colony stimulating factor (G-CSF), GRO/KC, vascular endothelial growth factor (VEGF), EGF, and Leptin], and anti-inflammatory cytokines [IL-10]. Out of the 27 measured analytes, four had concentrations outside the detectable range, resulting in 23 analytes for analysis. A heatmap was generated to visualize the concentrations of the 23 measured analytes (Fig. 5) and the corresponding concentration levels can be

found in supplementary Table S1. Distinct clustering patterns were observed, with an increase in Interferon gamma-induced protein (IP)-10 and Regulated on Activation, Normal T-cell Expressed and Secreted (RANTES) in Obese rats at 22-23w ($p_{IP-10}<0.01$; $P_{RANTES}<0.0001$) and 34-35w ($p_{IP-10}<0.0001$; $P_{RANTES}<0.05$). Furthermore, Monocyte Chemoattractant Protein (MCP)-1 was increased in Obese rats at 22-23w ($p<0.01$). Hierarchical clustering further highlighted similarities between Lean and Obese in cytokine and chemokine expression levels.

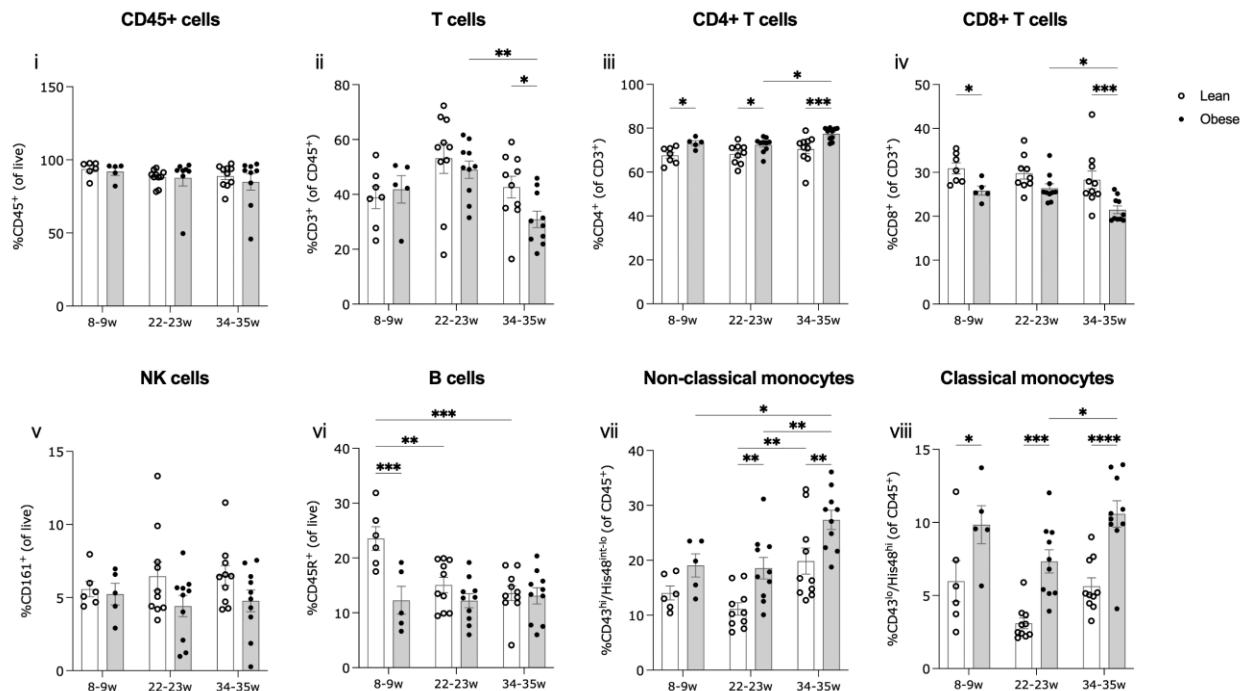


Figure 4. Circulating T-cell and monocyte populations are altered in Obese ZSF1 rats. Flow cytometric analysis in Obese and Lean rats at 8-9 weeks (8-9w), 22-23 weeks (22-23w) and 34-35 weeks (34-35w) of (i) CD45⁺ cells (2-way ANOVA: $p_{\text{page}}=0.3437$, $p_{\text{group}}=0.5362$, $p_{\text{page} \times \text{group}}=0.8863$), (ii) CD3⁺ T cells (2-way ANOVA: $p_{\text{page}}=0.0021$, $p_{\text{group}}=0.2311$, $p_{\text{page} \times \text{group}}=0.2668$), (iii) CD4⁺ T cells (2-way ANOVA: $p_{\text{page}}=0.0219$, $p_{\text{group}}<0.0001$, $p_{\text{page} \times \text{group}}=0.6266$), (iv) CD8⁺ cytotoxic T cells (2-way ANOVA: $p_{\text{page}}=0.0242$, $p_{\text{group}}<0.0001$, $p_{\text{page} \times \text{group}}=0.4273$), (v) NK cells (2-way ANOVA: $p_{\text{page}}=0.9507$, $p_{\text{group}}=0.0497$, $p_{\text{page} \times \text{group}}=0.6166$), (vi) B cells (2-way ANOVA: $p_{\text{page}}=0.0248$, $p_{\text{group}}=0.0007$, $p_{\text{page} \times \text{group}}=0.0105$), (vii) non-classical monocytes (2-way ANOVA: $p_{\text{page}}<0.0001$, $p_{\text{group}}=0.0002$, $p_{\text{page} \times \text{group}}=0.8106$), (viii) classical monocytes (2-way ANOVA: $p_{\text{page}}=0.0008$, $p_{\text{group}}<0.0001$, $p_{\text{page} \times \text{group}}=0.8139$). Data are presented as mean $\pm$ SEM. Tukey's multiple comparisons test: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. $n=5-10$ rats/group.

Furthermore, unsupervised Principal Component Analysis (PCA) was performed for each timepoint to identify patterns in cytokine expression across Lean and Obese groups (Fig. 5). The first principal component (PC1) accounted for most of the variance, followed by PC2, as shown in the scree plots for each timepoint (Supplementary Fig. 2). At 8-9w and 34-35w, the PCA plot did not show a clear separation between lean and obese groups along PC1 and PC2, as indicated by substantial overlap between the 95% confidence ellipses

around the means of each group. On the other hand, at 22-23w, the separation between Lean and Obese rats became more pronounced, with PC1 explaining 44.9% of the total variance and lean rats clustering more on the right side. The PCA loadings plots revealed which cytokines contributed most to the observed differences, with the top 10 most influential cytokines per timepoint shown in Suppl. Table 1. At 22-23w, pro-inflammatory cytokines such as IL-1a, IL-18, IL12p70, and IL-6 showed high

negative loadings on PC1, indicating their strong influence on the separation of Lean and Obese groups.

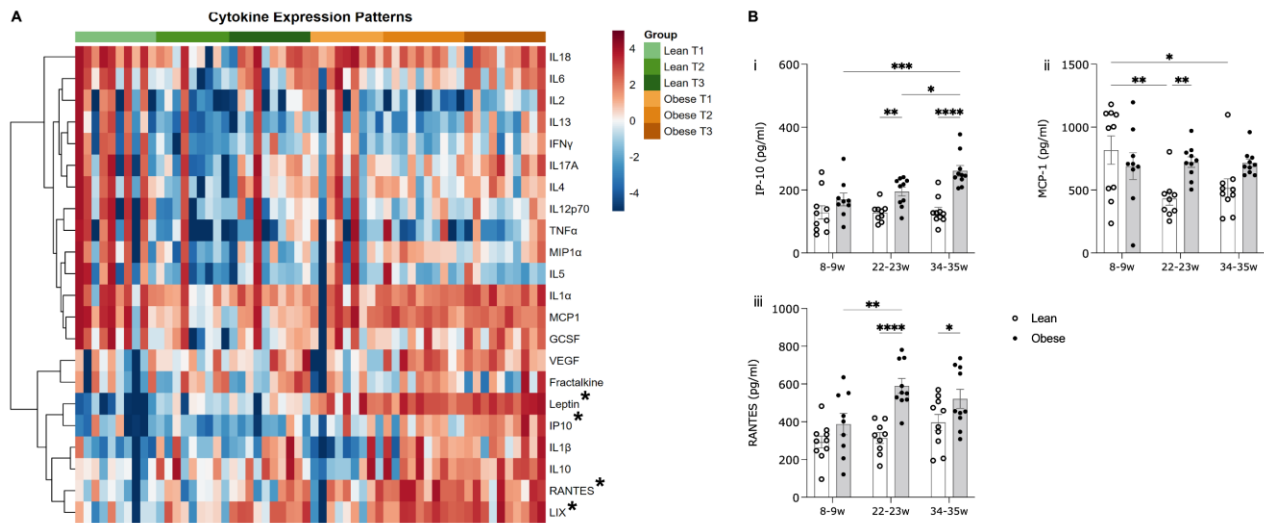


Figure 5. Plasma inflammatory mediator profiling reveals a time-dependent chemokine signature in Obese ZSF1 rats. (A) Heatmap of the plasma levels of cytokines and chemokines in Lean and Obese rats at 8–9 weeks (T1, 8–9w), 22–23 weeks (T2, 22–23w) and 34–35 weeks (T3, 34–35w). Different colors indicate different levels of protein expression, with blue to red representing low to high levels of expression. The dendrogram on the left side of the heat map indicates relationships between the expression profiles of the analysed cytokines across all of the samples assessed in this study. * indicates p_{group} . **(B)** Differential expression of a chosen set of cytokines regulated over time (i) IP-10 (2-way ANOVA: $p_{\text{age}}=0.0163$, $p_{\text{group}}<0.0001$, $p_{\text{age} \times \text{group}}=0.0278$), (ii) MCP-1 (2-way ANOVA: $p_{\text{age}}=0.0612$, $p_{\text{group}}=0.0571$, $p_{\text{age} \times \text{group}}=0.0217$), (iii) RANTES (2-way ANOVA: $p_{\text{age}}=0.0114$, $p_{\text{group}}<0.0001$, $p_{\text{age} \times \text{group}}=0.0857$). Data are presented as mean $\pm$ SEM. Tukey's multiple comparisons test: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. $n=9$ –10 rats/group.

Infiltrated and resident immune cells

To further investigate the role of different immune cell populations, resident and infiltrated immune cells were analyzed in kidneys and brains from lean and obese rats at all timepoints. At 8–9w, no signs of immune cell infiltration were observed in both kidney and brain of Lean and Obese rats. However, at 22–23w and 34–35w, an increase in infiltrating immune cells was found in the renal cortex of obese rats (Fig. 6).

Furthermore, the presence of macrophages was evaluated in the cortical area of kidney and brain of Lean and Obese rats (Fig. 6). An increase in CD68 $^{+}$ cells in the kidney was observed in obese compared to lean rats at 22–23w and 34–35w ($p<0.01$). While the number of CD68 $^{+}$ cells remained unchanged in lean rats over time, a significant increase in CD68 $^{+}$ cells was found in Obese rats at 22–23w 34–35w vs 8–9w ($p_{22-23w}<0.01$, $p_{34-35w}<0.05$). In the brain, a decrease in total CD68 $^{+}$ cells was observed in Obese vs Lean at 34–35w ($p<0.05$). Moreover, CD68 $^{+}$ cells increased from 8–9w to 34–35w in Lean rats ($p<0.01$). In addition, the number of perivascular macrophages (PVMs) also remained unchanged in both groups across timepoints (Supplementary Fig. 1).

We furthermore assessed the density and CD68 expression in microglia, the brain resident immune cells, in lean and obese rats over time (Fig. 7). While the number and surface area of microglia did not differ between Lean and Obese groups, a decreased density was observed in Lean rats over time at 34–35w vs 8–9w ($p<0.05$). In addition, we observed an overall decreased microglial surface area over time ($p<0.0001$) suggesting a shift from their homeostatic phenotype towards a more dystrophic or senescent phenotype. Quantification of phagocytic marker CD68 in microglia (CD68 $^{+}$ Iba1 $^{+}$) revealed a decrease in Obese vs Lean at 34–35w ($p<0.01$). Moreover, the number of CD68 $^{+}$ Iba1 $^{+}$ microglia increased in both Lean and Obese rats over time at 34–35w vs 8–9w ($p_{\text{lean}}<0.0001$, $p_{\text{obese}}<0.001$). However, CD68 $^{+}$ Iba1 $^{-}$ cells were decreased in Obese vs Lean ($p<0.05$) and a significant increase was observed in only Lean rats over time ($p<0.01$).

DISCUSSION

In the present study, we longitudinally characterized circulatory and tissue-specific inflammatory mediators associated with the progression of renal and cerebral dysfunction in a rat model of metabolic syndrome. This approach allowed us to compare systemic immune

changes with organ-specific responses over the course of disease progression. Importantly, our findings suggest that metabolic syndrome is not associated with a uniform inflammatory response across organs. Instead, Obese ZSF1 rats showed marked renal inflammatory and

macrophage-associated changes, whereas in the cerebral cortex, a decreased expression of CD68 was observed in microglia. These findings support the concept of divergent kidney and brain immune landscapes during metabolic syndrome.

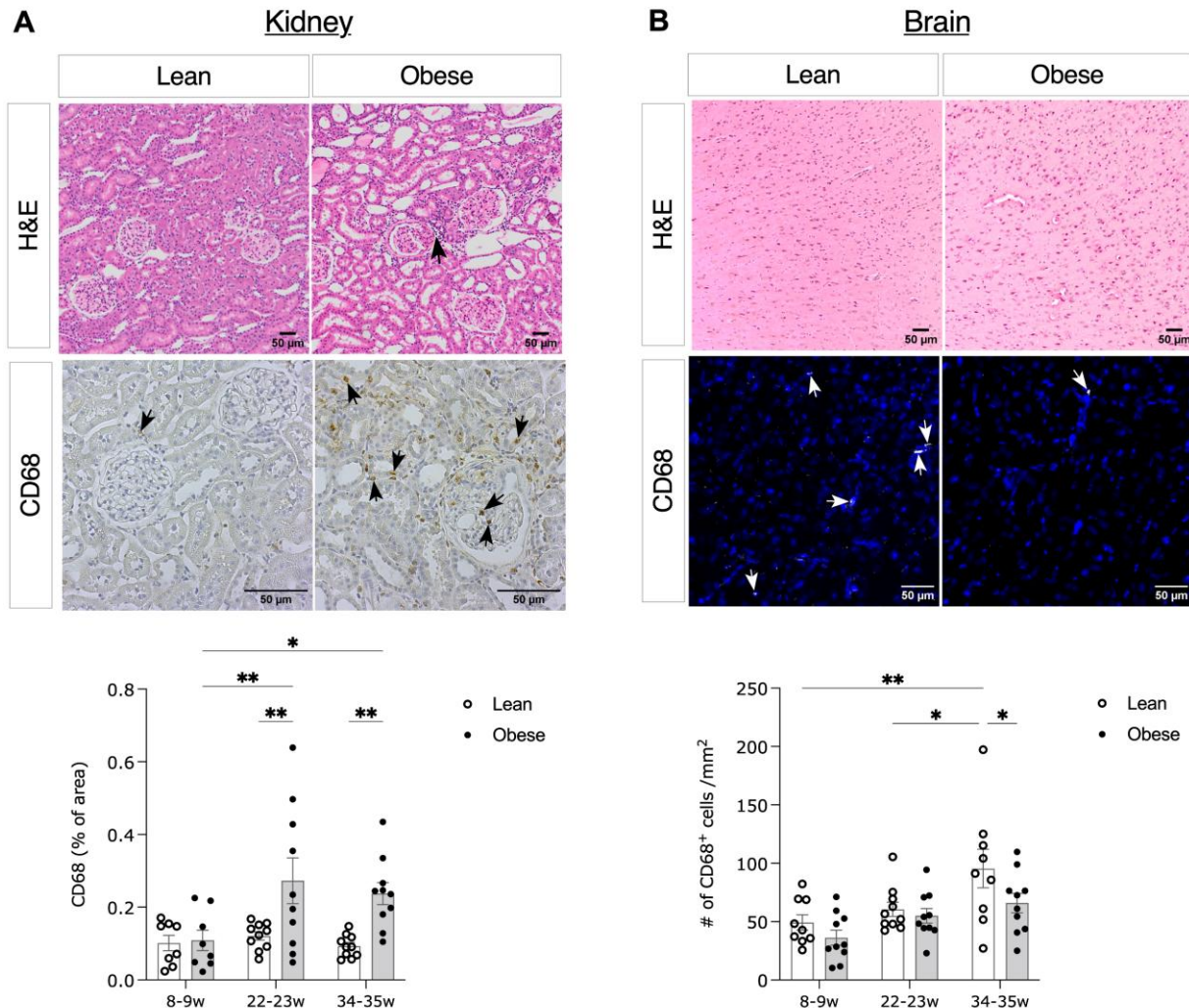


Figure 6. Obese ZSF1 rats display divergent kidney and brain CD68+ immune cell responses. Representative images of H&E (top) and CD68 stainings (bottom) in (A) renal cortex and (B) cerebral cortex in Lean and Obese rats at 22-23 weeks (22-23w). (A) The number of CD68+ cells (as % of area) in Lean and Obese kidneys at 8-9 weeks (8-9w), 22-23 weeks (22-23w) and 34-35 weeks (34-35w): CD68+ kidney macrophages (brown; black arrows) were quantified using CD68-immunoreactive areas (average area; 10 ROIs per section; 2 sections per animal) (2-way ANOVA: $p_{\text{age}}=0.0355$, $p_{\text{group}}=0.0006$, $p_{\text{age} \times \text{group}}=0.0784$); (B) The number of CD68+ cells (/mm²) in Lean and Obese brains at 8-9 weeks (8-9w), 22-23 weeks (22-23w) and 34-35 weeks (34-35w): CD68+ cells (white; white arrows) were segmented and quantified using a machine-learning-based pipeline (average count; 3 ROIs per section; 2 sections per animal) (2-way ANOVA: $p_{\text{age}}=0.0004$, $p_{\text{group}}=0.0317$, $p_{\text{age} \times \text{group}}=0.3892$). Data are presented as mean $\pm$ SEM. Tukey's multiple comparisons test: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. $n=9-10$ rats/group. Scale bar = 50 μ m.

Our findings confirm the presence of metabolic syndrome and kidney dysfunction in Obese ZSF1 rats, which is consistent with previous studies [11, 14]. Starting from 8-9w, Obese ZSF1 rats developed metabolic syndrome, characterized by hypertension and hyperglycemia. Renal histology demonstrated changes consistent with diabetic nephropathy, including tubular

dilation and atrophy, glomerular basement membrane thickening, and mesangial expansion from 22-23w. Moreover, an increase in kidney injury markers NGAL and KIM-1 as well as Collagen type III was observed in Obese rats at 22-23w and 34-35w, indicating further renal damage and fibrosis. In addition to the renal phenotype, cognitive dysfunction was observed in Obese ZSF1 rats

starting from 20 weeks of age in a longitudinal study by Lambrichts *et al.* [12]. In this study, obese animals had short-term memory impairment, altered spatial learning, and impaired long-term memory which could furthermore be linked to neurodegeneration, as obese rats also

demonstrated brain atrophy and lower white matter content [12]. Taken together, these observations indicate that the presence of multiple risk factors accompanies the onset of renal dysfunction and the development of cognitive impairment in Obese ZSF1 rats.

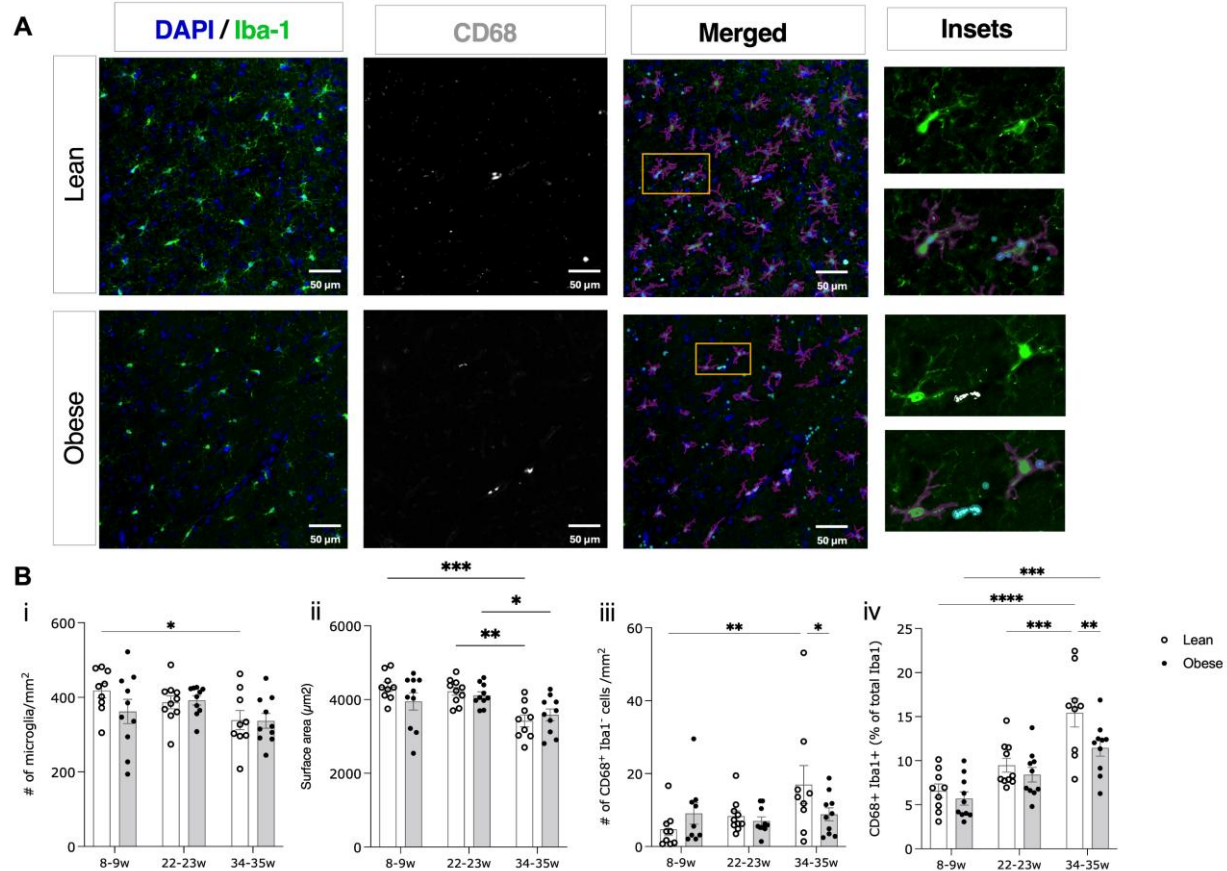


Figure 7. Obese ZSF1 rats show reduced late-stage CD68 expression in cortical microglia. (A) Representative images of Iba1/CD68 stain in the cortical area of Lean and Obese brains at 34–35 weeks (34–35w). Microglia were defined as Iba1-positive segmented objects (purple objects in merged images). CD68+Iba1+ microglia were defined as Iba1-positive microglial objects showing overlapping CD68 signal (cyan objects in merged images), identified using compartmentalization analysis after machine-learning segmentation. CD68+Iba1– cells were defined as CD68-positive objects without overlap with Iba1-positive microglia (average count; 3 ROIs per section; 2 sections per animal). The right column shows magnified insets of the boxed region indicated by the orange outline on the corresponding low-magnification image, displaying the segmented Iba1+ and CD68+ objects at higher resolution. (B) The number in Obese and Lean rats at 8–9 weeks (8–9w), 22–23 weeks (22–23w) and 34–35 weeks (34–35w) of (i) total microglia/mm² in the brain (2-way ANOVA: $p_{\text{age}}=0.0353$, $p_{\text{group}}=0.3412$, $p_{\text{age} \times \text{group}}=0.3307$), (ii) Microglial surface area in μm^2 (2-way ANOVA: $p_{\text{age}}<0.0001$, $p_{\text{group}}=0.36$, $p_{\text{age} \times \text{group}}=0.21$) (iii) CD68+ Iba1+ cells/mm² in the brain (2-way ANOVA: $p_{\text{age}}=0.0565$, $p_{\text{group}}=0.4301$, $p_{\text{age} \times \text{group}}=0.0740$), (iv) CD68+ Iba1+ cells (as % of total Iba1) in the brain (2-way ANOVA: $p_{\text{age}}<0.0001$, $p_{\text{group}}=0.0167$, $p_{\text{age} \times \text{group}}=0.2223$). Data are presented as mean $\pm$ SEM. Tukey's multiple comparisons test: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. $n=9$ – 10 rats/group. Scale bar = $50\mu\text{m}$.

An increasing body of evidence suggests that the link between kidney dysfunction and cognitive impairment is mediated through vascular mechanisms [15]. Intact kidney function is crucial for regulation of total blood volume and vascular tone [15]. Therefore, impairments in kidney function can lead to disturbances in regulation of blood flow in organs that are critically dependent on constant and adequate blood flow, such as the brain [2, 15]. In a population-based study, lower eGFR was

independently associated with lower cerebral blood flow [16]. Our findings revealed a decrease in renal blood flow in Obese rats while resting cerebral blood flow remained constant over time. The late reduction in renal blood flow likely reflects progressive renal vascular dysfunction in Obese ZSF1 rats. Importantly, renal histological abnormalities and increased kidney injury markers were already observed at 22–23 weeks, whereas the significant decrease in renal blood flow was detected at 34–35 weeks.

This suggests that reduced renal perfusion is unlikely to be the sole initiating driver of kidney injury in this model. It may represent instead a consequence of the progressive renal microvascular dysfunction, and kidney damage overall. Nevertheless, reduced renal blood flow may also contribute to further disease progression by aggravating local hypoxia, oxidative stress, and fibrosis, thereby acting as an amplifier of renal injury at later disease stages. Interestingly, this decreased renal perfusion contrasted with the preservation of resting cerebral blood flow. However, while baseline cerebral blood flow was unaltered, the study by Lambrechts *et al.* showed that Obese rats have impaired neurovascular coupling starting from 22–23w [12]. This was associated with an increase in vascular density in obese rats at 34–35w and increased vascular permeability [12]. These findings might reflect the induction of possible angiogenic processes as a compensatory and repair response to compensate for dysfunctional cerebral microvessels and to promote vessel repair. On the contrary, it has been suggested that the presence of multiple risk factors can lead to renal capillary rarefaction [17]. Capillary rarefaction may limit renal perfusion and oxygen delivery, thereby promoting the progression of CKD [17]. However, in the study by Nguyen *et al.*, capillary rarefaction was not observed in the kidney of ZSF1 rats at 26w [11]. Possibly, loss of capillaries develops at a later stage and is not yet present at 26w. However, these findings suggest that the loss of capillaries is not a prerequisite for the development of renal disease in the Obese ZSF1 rat [11].

To further elucidate the underlying mechanisms driving the renal and cerebral phenotype, we investigated systemic and organ-specific inflammatory mediators.

Among the mechanisms investigated, Nox emerged as a key contributor to oxidative stress in the kidney. Our findings showed an increase in Nox2 expression and NADPH Oxidase activity in the kidney of Obese rats at 22–23w and 34–35w. In contrast, no difference was observed in Nox4 expression between lean and obese rats at all timepoints. While Nox4 is more constitutively involved in processes like fibrosis, Nox2 primarily contributes to inflammatory Reactive Oxygen Species (ROS) production [18]. The lack of fibrotic tissue in kidneys of Obese ZSF1 rats could align with the absence of Nox4 upregulation. In addition, Nox2 is often expressed in macrophages [18]. Therefore, the increased Nox2 expression could originate from the increase in macrophages in obese kidneys. Moreover, increased levels of 8-Oxo-dG in urine of Obese rats indicate elevated oxidative DNA damage, which could potentially be linked to the upregulation of Nox2 and the overall oxidative stress environment in the kidney.

Furthermore, the presence of metabolic syndrome has been associated with systemic low-grade inflammation

[19]. We therefore quantified circulating immune cells by flow cytometry in Lean and Obese rats. At 22–23w, non-classical monocytes were increased in obese rats, while classical monocytes were increased in Obese rats at all timepoints. In another longitudinal study using the ZSF1 rat model, total pro-inflammatory and anti-inflammatory monocytes were increased in obese rats only at 21 weeks, which is partly in line with our findings [20]. Additionally, they reported a decrease in B cells in Obese rats at 6 weeks, which aligns with our findings showing decreased B cell levels in Obese vs Lean at 8–9w [20]. In our study, we observed a decrease in total T cells at 34–35w, and a decrease in cytotoxic T cells at 8–9w and 22–23w. In contrast, CD4⁺ T cells were increased in Obese rats at all timepoints. The study by Cuijpers *et al.* reported no difference in T cells and T helper cells at all timepoints [20]. Previous research has shown that peripheral T cells decrease with age accompanied by an increase in the ratio of CD4 to CD8 cells, which is in line with our results [21].

The role of immune cells in renal impairment processes has become very relevant, especially monocyte/macrophage function [22–24]. Initially during renal damage, macrophages can acquire a M1-like phenotype which favors a pro-inflammatory microenvironment [23]. On the other hand, as kidney disease progresses, these macrophages can polarize to a M2-like phenotype with an anti-inflammatory role promoting healing of damaged tissue [23]. Glomerulus cells may express chemoattractant chemokines which also promote the expression of cell adhesion molecules in macrophages. MCP-1 has been described as the main chemoattractant mediator of monocytes/macrophages during renal inflammation and is also related to diabetic nephropathy [25]. In our study, MCP-1 levels were increased in plasma of obese rats at 22–23w. More importantly, the systemic increase in MCP-1 corresponded with elevated MCP-1 expression in the kidney of Obese rats at 22–23w and 34–35w. Additionally, the distinction in cytokine expression in plasma between lean and obese groups was most evident, indicating that cytokine expression differs between groups at this timepoint. At 34–35w, the distinction between Lean and Obese groups became less pronounced, indicating more similarities in cytokine and chemokine expression possibly since Lean rats are hypertensive and develop similar inflammatory profiles over time.

The exact role of immune cells and key players was further investigated in kidney and brain tissues from Lean and Obese rats. While some immune cell infiltration was observed in kidneys from Obese rats at 22–23w and 34–35w, this was not the case for the brain. These findings align with the study by Deshpande *et al.* as they did not observe the presence of CD45^{high} infiltrating immune cells in the brain parenchyma of hypertensive BPH mice and

Notch3 mutant mice, suggesting that leukocyte extravasation is not a common feature of cSVD [26]. It should be noted, however, that the absence of macrophage infiltration does not rule out the presence of neuroimmune dysfunction in the brain of Obese ZSF1 rats. Neuroinflammatory and neurovascular processes can occur independently of peripheral leukocyte entry, for instance through endothelial activation, blood-brain barrier leakage, perivascular inflammation, astrocyte activation, complement activation, or shifts in microglial transcriptional state, none of which were assessed in the present study. In kidneys, an increase in CD68+ macrophages was observed in Obese vs Lean at 22-23w and 34-35w as well as an overall increase in macrophage number in Obese rats over time. This is in line with the findings by Nguyen *et al.*, who found a trend toward an increased number of glomerular CD68+ macrophages in Obese rats [11]. Similarly, in the study by Lavaud *et al.*, the number of glomerular macrophages was significantly higher in Obese male Zucker rats than in lean rats as early as one month of age [27]. On the contrary, a small but significant decrease in total CD68+ macrophages was observed in the cerebral cortex from Obese rats at 34-35w. In Lean rats, the number of CD68+ cells increased significantly over time. These findings suggest that the presence of obesity and diabetes, in addition to hypertension, elicits a distinct response in the brain compared to hypertension alone.

We further investigated the density and phenotype of resident brain immune cells, i.e. microglia. Microglia play a significant role in neuroinflammation and depending on the stage of the disease, microglia may act both protectively and detrimentally. In VCI, chemokines generated by endothelial cells attract microglia to the vessels, maintaining the integrity of the BBB. However, sustained inflammation may cause microglia to change from a protective to a deleterious phenotype [28]. However, the role of microglia in neuroinflammation within the pathogenesis of VCI remains unclear [28]. Therefore, we investigated the number and phenotype of microglia in the cerebral cortex of Lean and Obese rats. While the total number of microglia did not differ between groups, the number of microglia expressing the CD68 marker (CD68+ Iba1+) was decreased in Obese rats at the latest timepoint. Moreover, the increase in CD68+Iba1+ microglia over time was more pronounced in Lean vs Obese, again indicating a differential response in the presence of multiple risk factors. At the same time, CD68+ Iba1- cells- cells with monocytic origin- were decreased in Obese vs Lean at 34-35w. These findings suggest that metabolic dysfunction may alter the protective function of microglia, ultimately resulting in the cerebrovascular phenotype that has previously been reported [12].

Although renal injury and diabetic nephropathy have previously been described in Obese ZSF1 rats, the present study extends these observations by integrating renal pathology with longitudinal immune, inflammatory, and vascular readouts. Specifically, we combined circulating immune cell profiling, plasma cytokine/chemokine measurements, renal inflammatory and oxidative stress markers, renal blood flow assessment, histological evaluation, and CD68+ macrophage staining. This allowed us to place the established renal phenotype within a broader systemic and organ-specific immune context and to compare it with cerebral immune changes in the same metabolic syndrome model.

Taken together, while the renal inflammatory phenotype observed in Obese ZSF1 rats is consistent with CKD, this inflammatory progression was not paralleled in the brain, despite the presence of cognitive impairment at a later stage of disease. These findings suggest that metabolic syndrome is associated with divergent kidney and brain immune responses rather than a uniform inflammatory response across organs. This highlights the importance of investigating organ-specific immune landscapes to better understand the distinct pathological processes underlying CKD and VCI.

There are some limitations in our study that should be acknowledged. First, due to the study's descriptive nature, causal associations cannot be drawn. While we provided a comprehensive overview of immune cell dynamics and inflammatory responses, future research should elucidate the causal relationships between inflammatory markers and disease progression. Second, this study was performed exclusively in male ZSF1 rats, which represents an important limitation. Sex is a major biological variable in metabolic syndrome and its end-organ consequences, including renal disease, immune responses, neuroinflammation, and vascular cognitive impairment. In ZSF1 rats, Nguyen *et al.* reported sex-dependent differences in disease severity, with male Obese ZSF1 rats developing an earlier and more pronounced renal phenotype compared with female rats [12]. Therefore, the use of males only may have selected for a more severe metabolic and renal phenotype and may have influenced the magnitude or nature of the peripheral and cerebral immune alterations observed in the present study. This is particularly relevant for the interpretation of microglial readouts, as microglial phenotype and inflammatory responsiveness are known to differ between sexes and may evolve differently with age and disease. Accordingly, our conclusions should be interpreted as applying to male ZSF1 rats only. Future studies including both male and female animals will be required to determine whether similar organ-specific immune landscapes are present in female ZSF1 rats. Third, Obese ZSF1 rats serve as a valuable model for metabolic

syndrome, mimicking the human phenotype. However, the leptin receptor mutation may potentially influence immune responses, including microglia phenotype, which must be taken into consideration when interpreting inflammatory and immunological findings. Importantly, immune cell profiling was restricted to the cortical regions of the kidney and brain, potentially overlooking region-specific immune responses in other regions. To further characterize the phenotypic changes of microglia over time in this model, more refined morphological analyses, allowing discrimination between soma size and process complexity, together with deep transcriptomic profiling approaches, would be valuable in future studies. Finally, while Obese ZSF1 rats have been characterized in detail for impaired neurovascular coupling, increased BBB permeability, white matter changes, and cognitive impairment [12], the present study, together with this prior work, does not capture the full structural spectrum of human cSVD, including lacunes, cerebral microbleeds, enlarged perivascular spaces, or vessel wall remodeling. As with other preclinical models, the ZSF1 rat should therefore be regarded as capturing key functional and immune features relevant to cSVD and VCI, rather than as a complete structural model of human cSVD/VCI.

In conclusion, our longitudinal study revealed a systemic pro-inflammatory state in Obese ZSF1 rats, characterized by an increase in circulating monocyte subsets and inflammatory cytokines. Although macrophage infiltration was evident in the kidneys of obese rats, no such infiltration was observed in the brain, where instead a reduction in CD68 expression was observed in microglia in the cerebral cortex. Together, these findings suggest that the systemic immune challenge observed in this model of comorbidities leads to a differential immune response in the kidney and the brain, highlighting the importance of investigating organ-specific immune responses which may contribute to a better understanding of CKD and VCI pathophysiology.

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Supplementary Materials

The Supplementary data can be found online at: www.aginganddisease.org/EN/10.14336/AD.2026.0557.

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