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Innate Immunity

MRP8-Cre Transgenic Mice Are Resistant to Weight Gain and Diet-Induced Obesity Independently of Neutrophils

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ABSTRACT

Obesity is a major risk factor for metabolic syndrome, cancer, diabetes, and other diseases. The occurrence of comorbidities is associated with systemic and adipose tissue inflammation, which is marked by the presence of macrophages and neutrophils in adipose tissue. The role of neutrophils in driving adipose tissue inflammation and pathology is understudied and may hold therapeutic promise. *MRP8^{Cre}* transgenic mice are frequently used to investigate neutrophil functions. A recent report demonstrates that the insertion of the *MRP8^{Cre}* transgene resulted in whole-body deletion of *Ap1s1* and *Serpine1*, the latter encoding the pro-obesogenic factor plasminogen activator inhibitor-1 (PAI-1). Here, we show that *MRP8^{Cre}* transgenic mice have lower body weight than their littermate controls on a standard chow diet. Furthermore, they are resistant to weight gain, adipose tissue hypertrophy, systemic deregulation of glucose metabolism, infiltration of macrophages into AT, and liver steatosis upon feeding of obesogenic, high-fat diets (HFD). Mechanistically, this was independent of neutrophils or bone marrow-derived cells, sex, age of the mice, the duration of the HFD feeding, treatment start, housing facility, or the original source of the mice. The expression of *Serpine1*/PAI-1 and *Ap1s1* is globally reduced across metabolic organs of *MRP8^{Cre}* mice irrespective of diet, thus phenocopying previous reports of the obesity-protective effect of PAI-1 depletion in mice. Our findings demonstrate the limited applicability of *MRP8^{Cre}* mice for obesity or metabolic studies due to the observed off-target effects and highlight the need for respective controls when using transgenic mice, such as Cre-only controls or use of independent Cre lines for studies involving the Cre/loxP system.

1 | Introduction

Obesity is a major risk factor for type 2 diabetes, cancers, cardiovascular and thrombotic diseases. The etiology of the comorbidities has underlying contributions from chronic, sys-

temic low-grade inflammation, which originates from adipose tissue (AT) dysfunction during weight gain [1]. AT inflammation upon obesity arises from several factors, including adipocyte stress and death, mechanical stress on cells from surrounding fibrotic extracellular matrix (ECM), and dysregulation of fatty

Abbreviations: AT, adipose tissue; eWAT, epididymal white adipose tissue; iWAT, inguinal white adipose tissue; pgWAT, perigonadal white adipose tissue; HFD, high-fat diet.

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acid metabolism [2]. The inflammation hampers the metabolic state and mitochondrial function of the AT, adipogenesis and lipogenesis, which in turn causes ectopic lipid accumulation in the metabolic organs, such as the liver and skeletal muscle [2, 3]. Monocytes are recruited to the AT via locally released danger signals and chemokines such as CCL2, where they differentiate into AT macrophages, which are the key promoters of AT inflammation. The presence of macrophages in AT contributes to AT insulin resistance and subsequently to other downstream obesity-associated pathologies [2, 4]. Macrophage infiltration into AT is promoted by the presence of fibrin deposits and pro-coagulatory and antifibrinolytic molecules such as Factor XIII-A (FXIII-A) transglutaminase and PAI-1 in AT [5, 6]. The process, referred to as thromboinflammation, aggravates the obesity-related metabolic dysfunction and promotes weight gain [7, 8].

Neutrophils play a significant role in thrombotic disorders and AT inflammation, and elevated neutrophil numbers correlate with increased inflammation and worsening health [9–11]. Upon weight gain, neutrophils infiltrate AT within days, and initially drive AT inflammation via promoting pro-inflammatory macrophage recruitment, which further aggravates AT dysfunction and inflammation [12]. Furthermore, neutrophil-derived reactive oxygen species and neutrophil extracellular traps contribute to obesity-related inflammation [13, 14]. In line with this, reducing neutrophil abundance [15, 16], preventing neutrophil extracellular trap release [17] or inhibiting neutrophil elastase activity [18] improves the health status of obese mice fed a high-fat diet (HFD). Hence, a better understanding of the activities and biology of neutrophils as promoters of obesity-related pathologies holds great therapeutic promise.

Mouse models of obesity and transgenic mice represent invaluable tools for functional studies of the role of neutrophils in obesity, including neutrophil-specific gene deletion via the Cre-loxP system using *Lyz2^{Cre}* [19], *Ms4a3^{Cre}* [20], *Ly6g^{Cre}* [21], or *MRP8^{Cre}* [22] mice. *Lyz2^{Cre}* and *Ms4a3^{Cre}* driver lines delete genes in neutrophils and monocytes, which is suboptimal due to the known pathology-promoting role of monocyte-derived macrophages in obesity [2]. The *Ly6g^{Cre}* line is specific for neutrophils, but deletes very late in their development, which often only allows partial gene deletions in neutrophils that may be insufficient, especially during slowly progressing chronic disease processes such as obesity. In contrast, the *MRP8^{Cre}* line permits efficient gene deletion and is largely specific to neutrophils [23], which makes it well suited to study the relevance of neutrophils in obesity.

The transgenic *MRP8^{Cre}* mouse line was generated by the random genome integration of an *MRP8-Cre-ires-eGFP* cassette that mediates expression of the Cre recombinase and eGFP by the human S100A8 (myeloid-related protein 8, MRP8) promoter [22]. The *MRP8^{Cre}* mouse helped advance our knowledge on neutrophil biology, but limitations of this research tool have emerged [24, 25] as for other Cre lines [26]. Firstly, homozygosity for the *MRP8^{Cre}* transgene is lethal in mice, likely due to the insertion of the *MRP8-Cre-ires-eGFP* cassette into chromosome 5, which causes complete deletion of the *Serpine1* (Plasmin Inhibitor Activator-1, PAI-1) gene and partial deletion of *Apl1*

(Adaptor Related Protein Complex 1 Subunit Sigma 1) in the mouse genome [25]. Moreover, a recent report demonstrated that mice carrying the *MRP8^{Cre}* transgene develop arthritis, induced by anticardiolipin antibody, which was attributed to the lack of *Serpine1*/PAI-1 expression in neutrophils and macrophages upon LPS stimulation [24].

PAI-1 inhibits fibrinolysis as part of the hemostatic process to sustain blood clots, and its function is strongly linked to mediation of thromboinflammation and exacerbation of obesity-related pathologies, diabetes, and metabolic syndrome [7, 8, 27]. Systemic PAI-1 levels are elevated in obesity in patients and mice via increased expression in AT and liver [28–30]. Interference with PAI-1 in mice, either genetically or pharmacologically, leads to resistance to weight gain upon overnutrition or genetic predisposition. PAI-1 impairment also protects from AT inflammation and obesity-related metabolic dysfunctions [6, 31–34]. In human subjects, heterozygosity for *SERPINE1*/PAI-1 correlates with elevated lifespan and lower incidence of diabetes [35]. Currently, PAI-1 inhibitors are being developed for clinical applications in cancer, thrombosis, and age-related conditions (including obesity and diabetes) [36].

In this study, we report that *MRP8^{Cre}* transgenic mice have a decreased body weight, are resistant to diet-induced obesity, AT inflammation, liver steatosis, and the development of disturbances in diabetes/glucose metabolism. Expression of *Apl1* and *Serpine1* is significantly decreased in metabolic organs of *MRP8^{Cre}* transgenic mice fed with chow or an obesogenic diet, which likely causes the observed protection against diet-induced obesity. Our results emphasize the necessity for Cre-controls in research using gene editing via the Cre-loxP system in mice.

2 | Results

2.1 | *MRP8^{Cre}* Transgenic Mice Have a Reduced Body Weight and Are Protected From HFD-Induced Weight Gain Independent of Housing, Floxed Alleles or Bone Marrow-Derived Cells

We observed a decreased body weight of 8-week-old wildtype and heterozygous *MRP8^{Cre}* male and female littermates on a standard chow diet (Figure 1A). Additionally, to mimic an experimental scenario involving a Cre driver line for gene deletion, the Wculek laboratory used *MRP8^{Cre}* mice to eliminate a mitochondrial bioenergetics-related factor in neutrophils, the gene *Uqcrcq* [37]. Chow-fed male and female *MRP8^{Cre}* mice carrying *Uqcrcq^{f/f}* alleles (*MRP8^{Cre} Uqcrcq^{f/f}*) also had a lower body weight than their littermate *Uqcrcq^{f/f}* controls at 8 weeks of age, which was maintained over time (Figure 1B,C). Tibia length was equal or only slightly changed between experimental groups, suggesting largely unaltered growth of mice (Figure 1A). Next, to model diet-induced obesity, wildtype (*MRP8^{+/+}*) and heterozygous *MRP8^{Cre}* littermates as well as *Uqcrcq^{f/f}* and *MRP8^{Cre} Uqcrcq^{f/f}* littermates were placed on a 60% kcal from fat HFD, and their weight was monitored over time. Male and female *MRP8^{Cre}* mice showed a drastically reduced weight gain irrespective of the presence or absence of floxed alleles compared

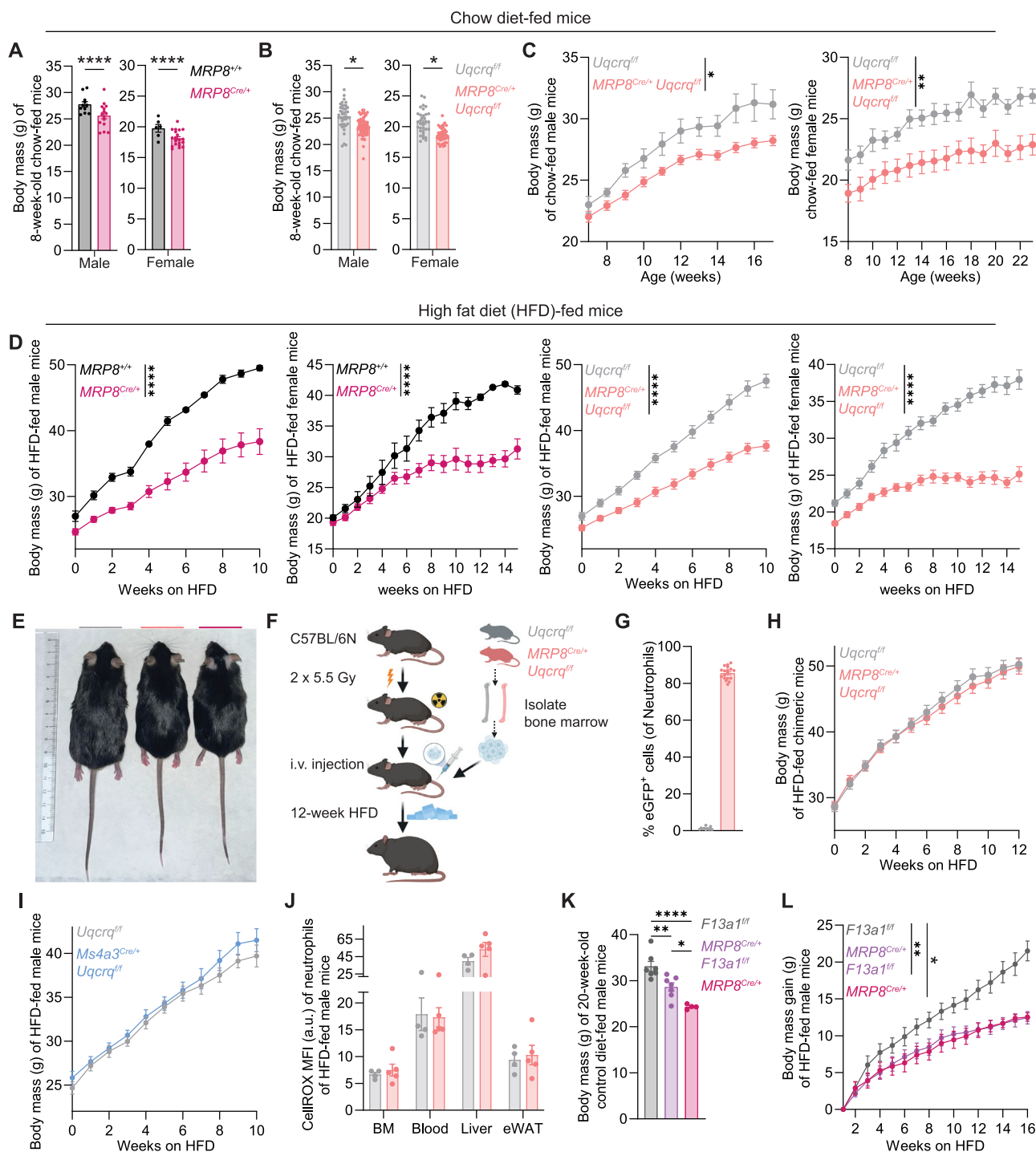


FIGURE 1 | *MRP8^{Cre}* transgene-carrying mice show reduced body mass and are protected from high-fat diet-induced obesity. (A) Body mass of 8-week-old chow-fed male and female *MRP8^{+/+}* and *MRP8^{Cre/+}* littermate mice ($n = 6-20$) and (B) *Uqcrcf^{fl/fl}* and *MRP8^{Cre/+} Uqcrcf^{fl/fl}* littermate mice ($n = 34-58$). (C) Body mass development of chow-fed male and female *Uqcrcf^{fl/fl}* and *MRP8^{Cre/+} Uqcrcf^{fl/fl}* mice ($n = 5-9$). (D) Body mass development of HFD-fed male and female *MRP8^{+/+}* and *MRP8^{Cre/+}* littermate mice ($n = 3-10$), or *Uqcrcf^{fl/fl}* and *MRP8^{Cre/+} Uqcrcf^{fl/fl}* littermate mice ($n = 8-20$). (E) Representative photographs of male *MRP8^{+/+}* and *MRP8^{Cre/+}*, *Uqcrcf^{fl/fl}*, and *MRP8^{Cre/+} Uqcrcf^{fl/fl}* mice after 10 weeks of HFD. (F) Experimental setup for generating *Uqcrcf^{fl/fl}* and *MRP8^{Cre/+} Uqcrcf^{fl/fl}* bone-marrow chimeric C57BL/6N mice shown in (G-H). Created in BioRender. Wculek, S. (2026) <https://BioRender.com/5ry5sqg>. (G) Frequency of *MRP8-Cre-eGFP* expressing (eGFP⁺) total CD11b⁺, Ly6G⁺ blood neutrophils in male C57BL/6N mice grafted 8 weeks prior with bone marrow of male *Uqcrcf^{fl/fl}* or *MRP8^{Cre/+} Uqcrcf^{fl/fl}* mice ($n = 11-18$). (H) Body mass development of HFD-fed male *Uqcrcf^{fl/fl}* and *MRP8^{Cre/+} Uqcrcf^{fl/fl}* bone-marrow chimeric C57BL/6N mice (HFD started 8 weeks post BM grafting, $n = 11-18$). (I) Body mass development of HFD-fed male *Uqcrcf^{fl/fl}* and *Ms4a3^{Cre/+} Uqcrcf^{fl/fl}* littermate mice ($n = 6-10$). (J) Flow cytometric analysis of cellular ROS levels in neutrophils from male *Uqcrcf^{fl/fl}* and *MRP8^{Cre/+} Uqcrcf^{fl/fl}* littermate mice after 10 weeks of HFD ($n = 4-5$). (K) Body mass of 20-week-old control diet-fed male *F13a1^{fl/fl}*, *MRP8^{Cre/+} F13a1^{fl/fl}*, and *MRP8^{Cre/+}* mice ($n = 4-7$). (L) Body mass development of HFD-fed male *F13a1^{fl/fl}*, *MRP8^{Cre/+} F13a1^{fl/fl}*, and *MRP8^{Cre/+}* mice ($n = 7$). Data are from at

with controls, which developed the expected increase in body mass (Figure 1D,E). Again, tibia length was largely equal or only minimally altered between experimental groups (Figure 1B). To dissect underlying causes of the observed weight gain protection of *MRP8^{Cre}* mice, we first interrogated the specific contribution of bone marrow-derived cells, mainly neutrophils and monocytes, as the *MRP8^{Cre}* transgene is globally inserted in all cells of *MRP8^{Cre}* mice. Male wildtype mice were lethally irradiated to ablate bone marrow cells and grafted with bone marrow from either control *Uqcrq^{f/f}* or *MRP8^{Cre} Uqcrq^{f/f}* littermates, which resulted in circulating neutrophil chimerism of about 90% (Figure 1F,G). Notably, *MRP8^{Cre}* transgene presence exclusively in bone marrow-derived cells did not reduce weight gain of mice upon HFD feeding (Figure 1H). This observation shows that factors other than neutrophils or bone marrow-derived cells underlie the weight-gain protection of HFD-fed *MRP8^{Cre}* mice. To further confirm this notion, we used *Ms4a3^{Cre}* mice to target gene expression in neutrophils [20]. Male mice carrying the *Ms4a3^{Cre}* together with our model floxed allele *Uqcrq^{f/f}* (*Ms4a3^{Cre} Uqcrq^{f/f}* mice) were not protected from HFD-induced weight gain either when compared with their littermates (Figure 1I; Figure 1SIC). In line with this, the abundance of neutrophils across tissues, their transcriptome, expression of maturation-associated proteins, respiratory burst capacity, and reactive oxygen species production was unaltered in chow-fed and/or HFD-fed control *Uqcrq^{f/f}* versus *MRP8^{Cre} Uqcrq^{f/f}* littermates (Figure 1J; Figure 1SID-I).

Secondly, we investigated a potential influence of other environmental or experimental factors on the decreased body weight and HFD-induced weight gain of *MRP8^{Cre}* mice, including the model floxed allele, age of starting HFD feeding, housing environment, and the origin of the *MRP8^{Cre}* mice. Therefore, similar studies were independently established by the Kaartinen group that bred *MRP8^{Cre}* mice with the floxed allele *F13a1^{f/f}* (*MRP8^{Cre} F13a1^{f/f}*) with the initial aim to investigate the role of fibrin crosslinking enzyme FXIII-A (*F13a1*) in neutrophils [5]. Chow-fed 20-week-old male *MRP8^{Cre}* and *MRP8^{Cre} F13a1^{f/f}* mice exhibited a decreased body weight compared with their *F13a1^{f/f}* controls (Figure 1K). Of note, full-body *F13a1*-deficient (*F13a1^{-/-}*) mice display features of metabolically healthy obesity upon obesogenic diet feeding and gain a comparable amount of weight as wildtype controls [5]. Next, male *MRP8^{Cre}*, *MRP8^{Cre} F13a1^{f/f}*, and control *F13a1^{f/f}* mice were fed a 60% HFD from 4 weeks of age onward. In line with our previous observations, the HFD-fed control mice showed a significant increase in body weight, but *MRP8^{Cre}* and *MRP8^{Cre} F13a1^{f/f}* mice were largely protected from HFD-induced weight gain (Figure 1L). A similar pattern was observed in female *MRP8^{Cre}* mice, with differences showing after 10 weeks on HFD (data not shown). Again, general growth and body size of *MRP8^{Cre}* mice remained unaltered compared with mice of other groups, as body length was similar (Figure 1IJ).

Overall, two independent laboratories observed that *MRP8^{Cre}* transgene heterozygous mice display a lower body weight on

standard chow diets than their control littermates and are resistant to weight gain on HFD. This phenotype was not influenced by *MRP8^{Cre}* transgene presence in bone marrow-derived cells/neutrophils, presence of floxed alleles, onset of HFD feeding, housing conditions, or source of *MRP8^{Cre}* mice, suggesting that the protection against HFD-induced obesity results from the global *MRP8^{Cre}* transgene insertion in those mice.

2.2 | *MRP8^{Cre}* Transgenic Mice Exhibit an Improved Global Metabolic Health Despite Equal Food Intake

Next, we analyzed parameters of systemic metabolic health, the pathophysiology of obesity, and the development of metabolic syndrome in control (*MRP8^{+/+}* or *Uqcrq^{f/f}*), *MRP8^{Cre}*, and/or *MRP8^{Cre} Uqcrq^{f/f}* mice on a chow diet and after HFD feeding. Chow-fed 16 (males) or 22 (females) week-old *Uqcrq^{f/f}* and *MRP8^{Cre} Uqcrq^{f/f}* littermates exhibited no changes in lean mass; especially, *MRP8^{Cre} Uqcrq^{f/f}* females had a slightly decreased fat mass, and both genders exhibited a trend towards accelerated clearing of injected high glucose in a glucose tolerance test (GTT) (Figure 2A,B). After HFD feeding, male and female *MRP8^{Cre}* heterozygous littermate mice had a significantly reduced fat mass by about 50% and only minimally unaffected lean mass (Figure 2C; Figure S2A), which suggests largely unaltered growth between experimental groups and a specific reduction in fat levels in *MRP8^{Cre}* mice. This was accompanied by significantly decreased plasma cholesterol and glucose concentrations in males and improved clearance of injected high glucose levels (GTT) in *MRP8^{Cre} Uqcrq^{f/f}* versus control littermates of both sexes (Figure 2D,E).

Of note, chow- and HFD-fed *MRP8^{Cre}* (and *MRP8^{Cre} Uqcrq^{f/f}*) mice consumed equal or elevated amounts of drink and food as controls (Figure 2F,G; Figure S2B-E), in line with their comparable plasma triglyceride concentrations after HFD feeding (Figure 2E). They exhibited similar voluntary movement activity, but especially female *MRP8^{Cre}* transgene-carrying mice showed an elevated energy expenditure compared with controls (Figure 2H,I; Figure S2B-E). This is likely contributing to the resistance of *MRP8^{Cre}* mice to HFD-induced weight gain.

Overall, the lower weight of *MRP8^{Cre}* transgenic mice on a chow diet and their reduced weight gain upon HFD feeding, specifically affecting fat mass, are not explained by alterations in food consumption and result in ameliorated systemic glucose metabolism.

2.3 | Reduced Fat Deposition Is Accompanied by Improved WAT and Liver Health in HFD-fed *MRP8^{Cre}* Transgenic Mice

Excess calories are stored in white adipose tissue (WAT), mainly epididymal/perigonadal (eWAT/pgWAT) and

least two independent experiments. Bars represent mean $\pm$ SEM. Dots represent individual data points. Statistical analysis by two-tailed unpaired *t*-test (A, B), one-way ANOVA (K), or two-way ANOVA (C, D, L). **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

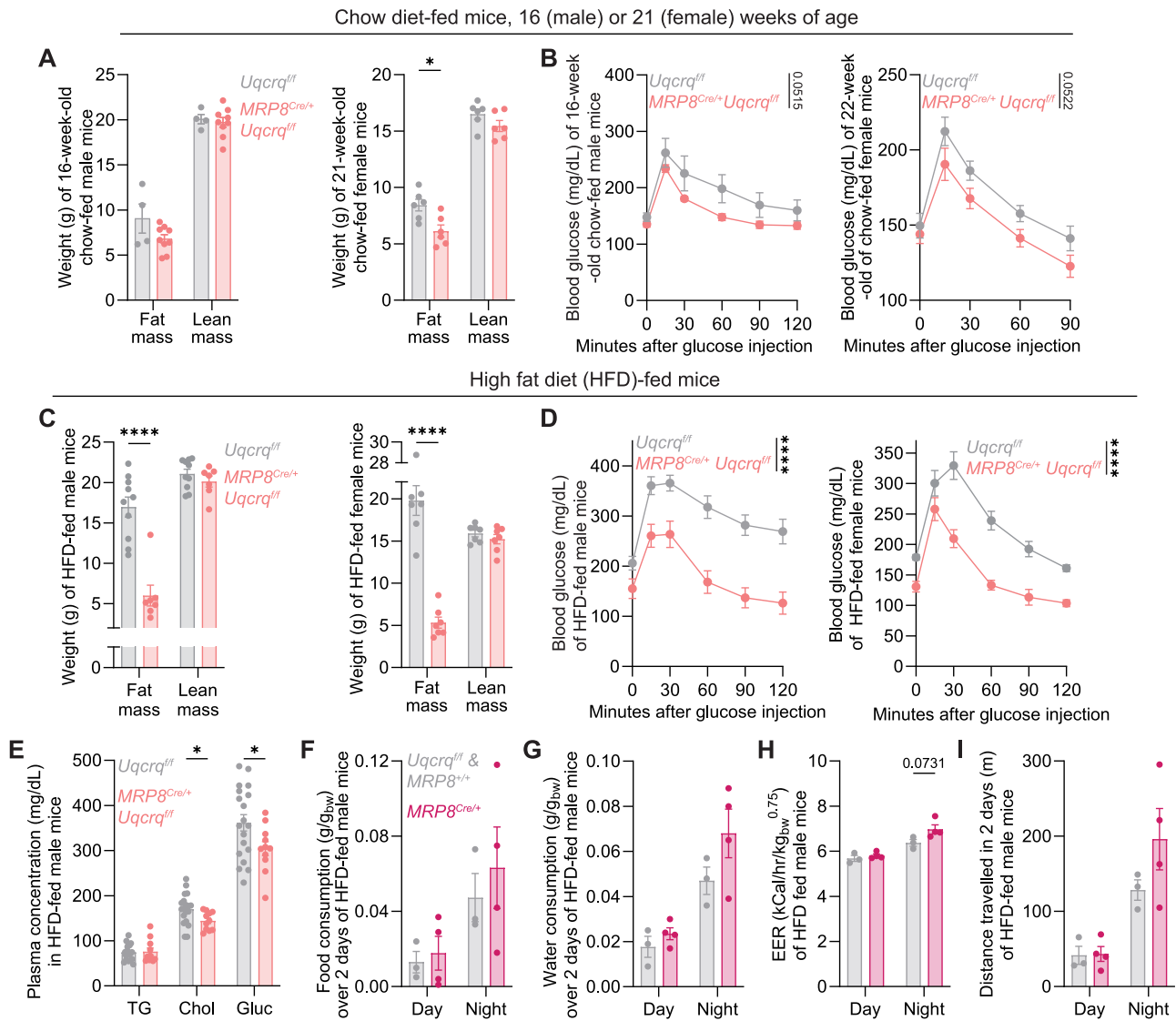


FIGURE 2 | Chow- and HFD-fed *MRP8^{Cre}* transgenic mice exhibit improved systemic metabolic features. (A) Body composition analysis of 16- or 21-week-old chow-fed male and female *Uqcrcf/f* and *MRP8^{Cre/+} Uqcrcf/f* mice by time-domain nuclear magnetic resonance (TD-NMR), respectively ($n = 4-9$). (B) Blood glucose concentration after intraperitoneal glucose injection of 16- or 21-week-old chow-fed male and female *Uqcrcf/f* and *MRP8^{Cre/+} Uqcrcf/f* mice, respectively ($n = 4-9$). (C) Body composition analysis of male and female *Uqcrcf/f* and *MRP8^{Cre/+} Uqcrcf/f* mice after 8 or 14 weeks of HFD by TD-NMR, respectively ($n = 7-10$). (D) Blood glucose concentration after intraperitoneal glucose injection in male and female *Uqcrcf/f* and *MRP8^{Cre/+} Uqcrcf/f* mice after 8 or 14 weeks of HFD, respectively ($n = 7$). (E) Plasma concentration of triglycerides (TG), cholesterol (Chol), and glucose (Gluc) of male *Uqcrcf/f* and *MRP8^{Cre/+} Uqcrcf/f* mice after 9 weeks of HFD ($n = 11-19$). (F-I) Metabolic and behavioral analysis assessed using a Promethion Core system over 2 days in *Uqcrcf/f* and *MRP8^{Cre/+} Uqcrcf/f* mice after 9 weeks of HFD ($n = 3-4$). (F) Food consumption. (G) Water consumption. (H) Average energy expenditure rate. (I) Distance travelled. Data are from at least two independent experiments. Bars represent mean $\pm$ SEM. Dots represent individual data points. Statistical analysis by two-tailed unpaired *t*-test with Holm-Šidák correction (A, C, E-I), or two-way ANOVA (B, D). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

inguinal/subcutaneous WAT (iWAT) fat pads, that become hypertrophic and inflamed upon obesity and, at more advanced stages, fat is deposited in the liver causing steatosis. WAT-associated inflammation is characterized by cytokine/chemokine production and elevated macrophage infiltration, which form crown-like structures to engulf dying adipocytes [1, 2, 4]. On a chow diet, the fat pads of *MRP8^{Cre}* transgenic mice had a mildly reduced weight compared with littermate controls, which was more pronounced upon age-related fat accumulation (Figure 3A; Figure S3A). However, the eWAT/pgWAT and iWAT fat pads were notably smaller in male and female HFD-fed *MRP8^{Cre}* and *MRP8^{Cre} Uqcrcf/f* mice

compared with control littermate mice (despite a low n number in one control group), with significantly smaller adipocytes in size in eWAT and iWAT (Figure 3B-E; Figure S3B,C). Furthermore, F4/80⁺ and CD64⁺ macrophage presence and crown-like structure formation were also reduced in eWAT and iWAT of male HFD-fed *MRP8^{Cre}* mice, in concert with decreased expression of the monocyte-attracting chemokine *Ccl2* (Figure 3D,F; Figure S4A-G). Yet, the expression of other inflammatory parameters in eWAT/iWAT, WAT neutrophil infiltration, or systemic/circulating monocyte and neutrophil numbers remained unaffected (Figures S4H and S4F,J). HFD-fed female *MRP8^{Cre}*

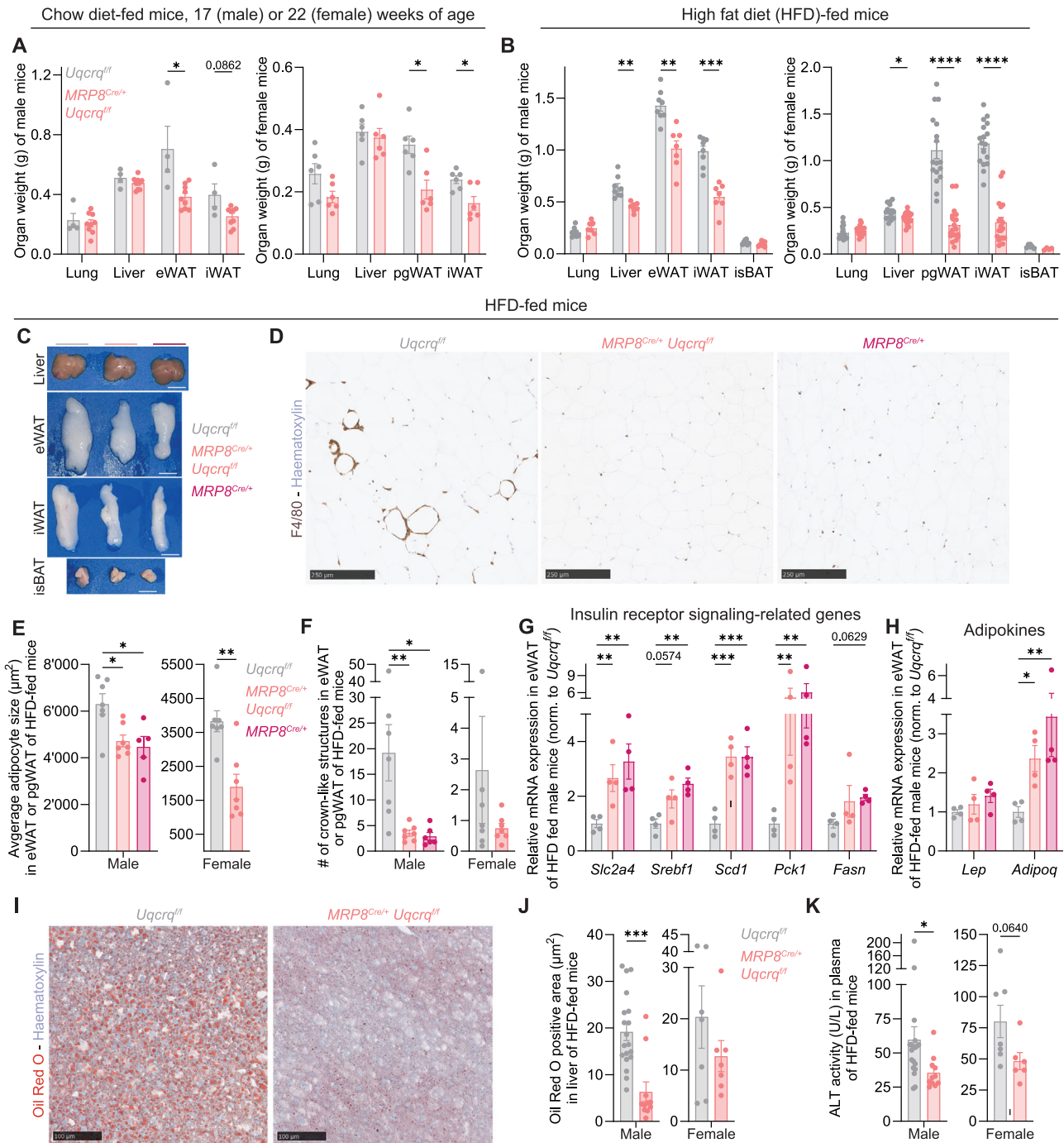


FIGURE 3 | *MRP8^{Cre}* transgene-carrying mice show reduced fat deposition and improved white adipose tissue and liver health during HFD feeding. (A) Organ weights of chow-fed 17- or 23-week-old male and female *Uqcrcf/f* and *MRP8^{Cre/+} Uqcrcf/f* littermate mice, respectively ($n = 4-9$). Epididymal white adipose tissue (eWAT), inguinal WAT (iWAT), and perigonadal WAT (pgWAT). (B) Organ weights of male and female *Uqcrcf/f* and *MRP8^{Cre/+} Uqcrcf/f* after 10 or 15 weeks of HFD, respectively ($n = 7-19$). Interscapular brown adipose tissue (isBAT). (C) Representative photographs of organs from HFD-fed male mice. Scale bar represents 1 cm. (D) Representative microscopy images of sections of eWAT from HFD-fed male mice stained for F4/80 (brown) and hematoxylin (blue). Scale bar represents 250 μm. (E) Quantification of average adipocyte size in eWAT or pgWAT across 5 regions of interest (ROIs) in male *Uqcrcf/f*, *MRP8^{Cre/+} Uqcrcf/f*, and *MRP8^{Cre/+} Uqcrcf/f* mice after 10 weeks of HFD, or female *Uqcrcf/f* and *MRP8^{Cre/+} Uqcrcf/f* mice after 15 weeks of HFD ($n = 5-7$). (F) Number of crown-like structures (CLS) in eWAT or pgWAT across 5 ROIs in male *Uqcrcf/f*, *MRP8^{Cre/+} Uqcrcf/f*, and *MRP8^{Cre/+} Uqcrcf/f* mice after 10 weeks of HFD, or female *Uqcrcf/f* and *MRP8^{Cre/+} Uqcrcf/f* mice after 15 weeks of HFD ($n = 6-7$). (G) Relative mRNA expression of insulin receptor signaling-related genes and (H) adipokines in eWAT of male *Uqcrcf/f*, *MRP8^{Cre/+} Uqcrcf/f*, and *MRP8^{Cre/+} Uqcrcf/f* mice fed a HFD for 10 weeks ($n = 4$). (I) Representative microscopy images of liver from HFD-fed male mice stained for Oil Red O (red) and hematoxylin (blue). Scale bar represents 100 μm. (J) Average Oil Red O-stained area across 5 ROIs in male *Uqcrcf/f*, *MRP8^{Cre/+} Uqcrcf/f*, and *MRP8^{Cre/+} Uqcrcf/f* mice after 9 weeks of HFD, or in female *Uqcrcf/f* and *MRP8^{Cre/+} Uqcrcf/f* mice after 15 weeks of HFD ($n = 7-19$). (K) Activity of liver enzymes alanine aminotransferase (ALT) in plasma of male *Uqcrcf/f*, and *MRP8^{Cre/+} Uqcrcf/f* mice after 15 weeks of HFD ($n = 7-19$).

Uqcrcq^{f/f} versus littermate *Uqcrcq^{f/f}* mice did not exhibit altered macrophage presence in pgWAT (Figure 3F; Figure S4B,E). Furthermore, work in the Kaartinen laboratory confirmed that the elevated macrophage infiltration into eWAT and iWAT upon HFD feeding of control *F13a1^{f/f}* mice was also not observed in male *MRP8^{Cre} F13a1^{f/f}* mice by analyzing expression of the macrophage-characteristic gene *Adgre1* that encodes F4/80 (Figure S4K). Given the pronounced protection of *MRP8^{Cre}* transgene-carrying mice from overnutrition-induced morphological alterations of the WAT, but only mild changes in inflammation that predominantly affect macrophage presence, we further explored parameters of WAT health in those mice. First, insulin-sensitive genes and genes characteristic of insulin (receptor) signaling were more highly expressed in eWAT of male HFD-fed *MRP8^{Cre}* and *MRP8^{Cre} Uqcrcq^{f/f}* mice versus control mice/littermates (Figure 3G), suggesting improved insulin sensitivity of WAT. Similarly, *Adipoq* (encoding adiponectin) expression, but not that of *Lep* (encoding leptin), was elevated in eWAT of male *MRP8^{Cre}* mice on a HFD (Figure 3H), which further indicates ameliorated metabolic health of the WAT of HFD-fed *MRP8^{Cre}* transgenic mice. Finally, despite a mildly altered expression of genes involved in thermogenesis, male HFD-fed *MRP8^{Cre}* and *MRP8^{Cre} Uqcrcq^{f/f}* littermates exhibited a reduced expression of the central WAT beiging regulator *Dio2* and undetectable expression of the thermogenesis factor UCP1 across genotypes in the iWAT (Figure S5A,B), a tissue with a known role in adaptive thermogenesis [38]. Overall, those data suggest similar inflammatory gene expression in WAT, despite decreased WAT macrophage infiltration and ameliorated WAT insulin sensitivity, adipocyte function, and improved metabolic health in HFD-fed *MRP8^{Cre}* transgenic mice compared with controls, with no overt signs of WAT beiging.

Next, we investigated indicators of steatosis and observed unaltered liver weights in chow-fed *MRP8^{Cre}* versus *MRP8^{+/+}* and *MRP8^{Cre} Uqcrcq^{f/f}* versus *Uqcrcq^{f/f}* littermates (Figure 3A; Figure S3A). Yet, livers of foremost male *MRP8^{Cre}* and *MRP8^{Cre} Uqcrcq^{f/f}* mice after HFD feeding remained lighter than those of their littermates (Figure 3B; Figure S3B), despite a low *n* number in one control group. Indeed, male *MRP8^{Cre} Uqcrcq^{f/f}* mice showed significantly decreased lipid accumulation in the liver and reduced concentrations of the lipid damage-associated protein alanine aminotransferase (ALT) in plasma (Figure 3I–K). Female *MRP8^{Cre}* transgenic mice only showed very mildly ameliorated liver health parameters, likely due to the known protection of females against HFD-induced liver pathologies (Figure 3J,K). The weight of nonmetabolic organs such as the lung did not seem to be overtly affected in *MRP8^{Cre}* mice irrespective of sex or diet (Figure 3A,B; Figure S3A,B).

In summary, we found that mice carrying the *MRP8^{Cre}* transgene are largely resistant to HFD-induced adipose tissue hypertrophy, likely due to improved AT physiology, including insulin signaling, which also elevates their liver health.

2.4 | Expression of *Serpine1* (PAI-1) Is Decreased Across Metabolic Organs of *MRP8^{Cre}* Transgenic Mice Upon HFD Feeding

The insertion of the *MRP8^{Cre}* transgene into the mouse genome causes a heterozygous, global deletion of *Serpine1* (PAI-1) and *Ap1s1* [25] (Figure 4A), which mimics *SERPINE1* heterozygous loss of function in human subjects that have a decreased prevalence of diabetes [35]. We queried the CZ CELLxGENE Discover platform [39] for expression of *SERPINE1* across distinct cell types in human liver and adipose tissue and found it to be ubiquitously expressed without clear tissue or cell tropism (Figure S5C,D). Hence, we evaluated the expression of *Serpine1* and *Ap1s1* in *MRP8^{Cre}* mice in fat depots. Firstly, as expected [28–30], we observed that *Serpine1* expression is significantly upregulated in iWAT of control *F13a1^{f/f}* mice upon HFD feeding compared with control diet-fed *F13a1^{f/f}* mice, although this was not observed in eWAT. *Ap1s1* was also upregulated in the iWAT and eWAT of control *F13a1^{f/f}* mice upon HFD feeding (Figure 4B,C). Notably, we found a significantly reduced expression (by more than 50%) of *Serpine1* and *Ap1s1* in fat pads of chow- and HFD-fed *MRP8^{Cre} F13a1^{f/f}* versus control *F13a1^{f/f}* mice (Figure 4B,C). In line, *Serpine1* and *Ap1s1* expression was also significantly decreased in eWAT, iWAT and liver from chow- and HFD-fed male and female *MRP8^{Cre}* and *MRP8^{Cre} Uqcrcq^{f/f}* mice compared with control/littermate *Uqcrcq^{f/f}* mice (Figure 4D–G).

PAI-1/*Serpine1* impairment was shown to decrease adiposity, reduce weight gain and obesity-related pathologies in mice and humans [6, 31–35]. However, pro-obesogenic effects of *Serpine1* loss in mice were also reported [34]. The genetic background of mice seems to account for those contradictory findings [40], which will need to be considered in translational studies of PAI-1/*Serpine1* in metabolic diseases. Notably, PAI-1/*Serpine1* loss in adipocytes improves adipocyte insulin sensitivity and glucose uptake, and PAI-1/*Serpine1*-deficient adipocytes are resistant to TNF α -induced dedifferentiation and insulin signaling inhibition [41]. Hence, the significant decrease of *Serpine1* expression across metabolic organs of HFD-fed *MRP8^{Cre}* transgenic mice is likely contributing to their resistance to the gain of fat mass, AT hypertrophy and deterioration, macrophage infiltration into AT, and associated systemic metabolic syndrome. On the other hand, homozygous deficiency of *AP1S1*/*Ap1s1* is embryonically lethal in mice and humans [42, 43], which hampers studies of its function. However, permissive pathogenic variants of *AP1S1* are associated with a rare autosomal recessive neurocutaneous disorder called IDENNIK that can cause diarrhea, liver disease, and mental and physical retardation [44]. Interestingly, the complete loss of the related isoform *Ap1s2* in mice was implicated in impaired adipocyte differentiation, but does not affect systemic glucose homeostasis [42]. Hence, *Ap1s1* loss and/or the simultaneous loss with *Serpine1* may additionally influence the WAT, liver and systemic metabolic health of *MRP8^{Cre}* transgenic mice.

MRP8^{Cre/+} Uqcrcq^{f/f}, and *MRP8^{Cre/+}* mice after 9 weeks of HFD, or of female *Uqcrcq^{f/f}* and *MRP8^{Cre/+} Uqcrcq^{f/f}* mice after 15 weeks of HFD (*n* = 6–19). Data are from at least two independent experiments. Bars represent mean $\pm$ SEM. Dots represent individual data points. Statistical analysis by two-tailed unpaired *t*-test (E, F, J, K) with Holm–Šidák correction (A, B), or one-way ANOVA with Tukey correction (E–H). **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

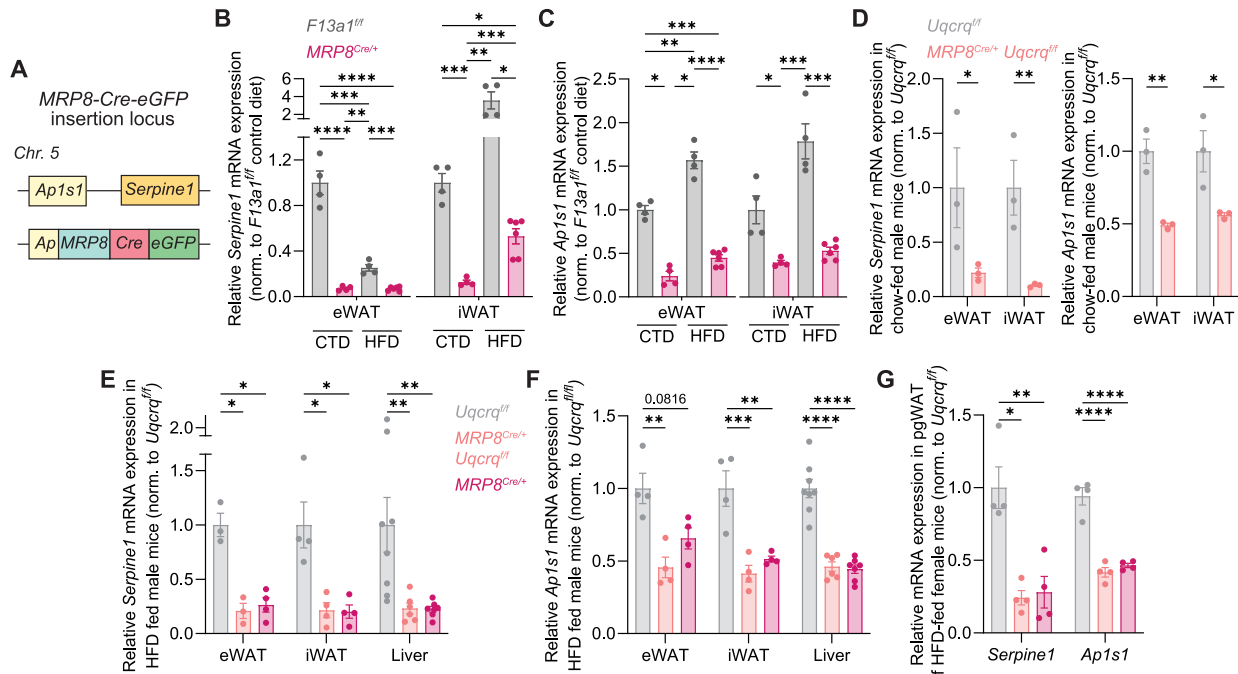


FIGURE 4 | The expression of the pro-obesogenic factor *Serpine1*/PAI-1 is reduced in metabolic tissues of chow- and HFD-fed *MRP8^{Cre}* transgenic mice. (A) Schematic of the *MRP8-Cre/IRES-eGFP* transgene and its genomic integration locus on mouse chromosome (Chr.) 5 (heterozygous), as described previously [25]. (B, C) Male *F13a1^{fl/fl}*, *MRP8^{Cre/+} F13a1^{fl/fl}*, and *MRP8^{Cre/+}* mice were fed a nutrition-matched control diet (CTD) or high-fat diet (HFD) for 16 weeks. Relative mRNA expression of *Serpine1* (left panel) and *Aps1s1* (right panel) in eWAT and iWAT ($n = 4-6$). (D) Relative mRNA expression of *Serpine1* (left panel) and *Aps1s1* (right panel) in chow-fed 17-week-old male *Uqcrcq^{fl/fl}* and *MRP8^{Cre/+} Uqcrcq^{fl/fl}* mice ($n = 3-4$). (E, F) Relative *Serpine1* (E) and *Aps1s1* (F) mRNA expression in eWAT, iWAT, and liver of male *Uqcrcq^{fl/fl}*, *MRP8^{Cre/+} Uqcrcq^{fl/fl}*, and *MRP8^{Cre/+}* mice after 10 weeks of HFD feeding ($n = 3-8$). (G) Relative *Serpine1* and *Aps1s1* mRNA expression in pgWAT and iWAT female *Uqcrcq^{fl/fl}*, *MRP8^{Cre/+} Uqcrcq^{fl/fl}*, and *MRP8^{Cre/+}* mice after 15 weeks of HFD feeding ($n = 4$). Data are from at least two independent experiments. Bars represent mean $\pm$ SEM. Dots represent individual data points. Statistical analysis by two-tailed unpaired *t*-test with Holm-Šidák correction (D), one-way ANOVA with Tukey correction (E-G), or two-way ANOVA with Tukey correction (B, C). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3 | Discussion

In this study, we report that *MRP8^{Cre}* transgenic mice have an intrinsic resistance to weight gain on normal diets, which is aggravated upon overnutrition-induced metabolic stress. We observed reduced expression of *Serpine1*/PAI-1 and *Aps1s1* in their fat depots and other metabolic organs, which is likely explained by the heterozygous loss of both genes due to *MRP8^{Cre}* transgene integration [25]. *MRP8^{Cre}* mice were protected from weight gain, AT macrophage infiltration, liver steatosis, and diabetes upon HFD feeding, which phenocopies PAI-1 impairment in obese mice [6, 31–34] and PAI-1 heterozygosity in human subjects [35]. Notably, the improved metabolic health of *MRP8^{Cre}* transgenic mice seems to be largely equal in both sexes and independent of floxed alleles, housing conditions, onset of HFD feeding, and, especially, neutrophils. The decreased global expression of *Serpine1* in chow- and HFD-fed male and female *MRP8^{Cre}* transgenic mice seems plausible to account for their improved metabolic health. However, additional factors may influence or promote the intrinsic (metabolic) phenotypes of *MRP8^{Cre/+}* mice, including the decreased expression of *Aps1s1* [42], off-target effects of Cre or eGFP expression, alterations in tertiary DNA structure due to transgene integration, and other unknown variables, which remain to be defined. Importantly, *Serpine1*/PAI-1 and *Aps1s1* play crucial roles beyond obesity or metabolic syndrome, including inflammatory diseases, cancer, cerebral infarction, T

cell responses [35, 45, 46], and other rare diseases such as IDEDNIK, respectively [44, 47, 48]. Hence, we recommend caution when using the *MRP8^{Cre/+}* transgenic mouse model in experimental research across different fields and diseases, which is highlighted by their recently described susceptibility to arthritis [24].

In conclusion, our study advocates for the careful evaluation of *MRP8^{Cre}* transgenic mice for studies of metabolism, thrombosis, or any context where *Serpine1*/PAI-1 or *Aps1s1* may be involved. On a broader scale, our findings emphasize the importance of including appropriate controls in experiments using genetic mouse models, foremost the necessity of Cre-only controls and the validation of experimental results in independent Cre lines by studies relying on the mouse Cre-*loxP* system.

4 | Materials and Methods

4.1 | Mice

C57BL/6N wildtype, *B6.Cg-Tg(S100A8-cre-EGFP)Ilw* (*MRP8^{Cre}* kind gift from A. Hidalgo [CNIC, Madrid, Spain] used by the Wculek lab or obtained from The Jackson Laboratory [stock #021614] use by the Kaartinen lab), *Uqcrcq^{L1Ncdl} (Uqcrcq^{fl/fl})* [37],

Ms4a3^{em2(cre)Fgmx} (*Ms4a3^{Cre}* kind gift from F. Ginhoux; Gustave Roussy, Villejuif, France) [20] and *FXIII-A^{flox/flox}* (*FI3a1^{f/f}*) [49] mice were kept in specific pathogen-free conditions with 12 hour light–dark cycle and ad libitum access to food and water at the animal facility of the Parc Científic de Barcelona (registry number B9900044) or in the Animal Facility of McGill University. All *Uqcrq^{f/f}* and *MRP8^{Cre/+} Uqcrq^{f/f}* mice, as well as all used *MRP8^{+/+}* and *MRP8^{Cre/+}* mice in experiments, were always littermates. Additionally, we included age- and sex-matched *MRP8^{Cre/+}* mice in several analyses using *Uqcrq^{f/f}* and *MRP8^{Cre/+} Uqcrq^{f/f}* littermates or *FI3a1^{f/f}* mice.

4.2 | Diet-Induced Obesity, Metabolic Assessments and Mouse Procedures

Eight-week-old male and female mice received 60% kCal from fat HFD (D12492i, ResearchDiets Inc.) for 9–10 (males) or 15 (females) weeks at IRB Barcelona or standard chow diet (U8100G10R, SAFE Diets). Four-week-old male and female mice received 60% kCal from fat HFD (TD.06414, Inotiv Inc) for 16 weeks, and control mice received nutrition-matched 10% kCal from fat control diet (TD.08806, Inotiv Inc) at McGill University.

For Glucose tolerance tests, fasted mice were injected intraperitoneally with 1 g/kg glucose. Blood glucose concentration was determined using an Accu-Chek Instant (Roche). Body composition was assessed using a minispec LF50 Whole Body Composition Analyzer (Bruker). Metabolic phenotyping was performed using a Promethion Core (Sable Systems). For bone marrow transplantation, male recipient C57BL/6N mice received two lethal X-ray radiation doses of 5.5 Gy (SMART 200, RX-YLON International) before intravenous injection of 20×10^6 donor bone marrow cells from *Uqcrq^{f/f}* and *MRP8 Δ Uqcrq* mice.

4.3 | Tissue Dissociation for Cell Isolation

Bone marrow was isolated from the tibia and femurs by cutting open the epiphyses and flushing the bone marrow with ice-cold PBS. Blood was collected in EDTA K3 microvette tubes (Sarstedt). Red blood cells were lysed with red blood cell lysing buffer Hybri-Max (R7757, Sigma-Aldrich), and cells were filtered through a 70 μ m cell strainer.

Mice were perfused with HBSS (11560456, Gibco). eWAT and pgWAT were minced and incubated for 20 min in HBSS (Gibco) with 0.5% BSA (A7030, Merck) and 2 mg/mL Collagenase II (LS004176, Worthington) at 37°C and shaking at 250 rpm. Digested tissue was filtered through a 100 μ m cell strainer. Red blood cells were lysed using red blood cell lysing buffer Hybri-Max and cells filtered through a 40 μ m cell strainer.

4.4 | Oxidative Burst

Bone marrow cells were isolated as stated above and incubated with biotinylated anti-Ly6G antibody in MACS buffer (130-091-221, Miltenyi Biotec) for 20 min on ice. Cells were

washed and incubated with Streptavidin Microbeads (130-048-102, Miltenyi Biotec) for 15 min on ice. Cells were washed and positively labelled cells separated using an LS column (130-042-401, Miltenyi Biotec) as per the manufacturer's instructions. The neutrophil-enriched fraction (>95% purity) was resuspended in Seahorse media (103576-100, Agilent) supplemented with 10 mM glucose (15384895, ThermoFisher), 1 mM pyruvate (11530396, ThermoFisher), and 2 mM glutamine (11574466, ThermoFisher). Seahorse plates (102340-100, Agilent) were coated with 22.4 μ g/mL Cell-Tak (354240, Corning), and 1.5×10^6 neutrophils were plated in each well. The plate was centrifuged at 50 $\times$ g for 30 s without brake to attach floating neutrophils to the bottom of each well. After a 45 min incubation at 37°C, the plate was placed into a Seahorse XFe24 (Agilent) and the assay started. To induce oxidative burst, neutrophils were stimulated with 500 nM PMA (P1585, Sigma-Aldrich) through a Seahorse well plate port.

4.5 | Flow Cytometry

Single cell suspensions were incubated for 20 min at 4°C in PBS supplemented with 2% FBS (A5256801, ThermoFisher) and 2.5 mM EDTA (A3145.1000, ITW Reagents) containing Fc Shield (70-0161-M001, Cytok Biosciences) and a mix of fluorochrome-conjugated antibodies. Dead cells were stained with 0.1 μ g/ μ L DAPI (D1306, ThermoFisher) before acquisition on a FACSARIA Fusion or FACSsymphony A3 (BD Biosciences). A representative gating strategy is shown in Figure S6. Antibodies used for flow cytometry are shown in Table S1.

To measure cellular ROS levels, antibody-stained neutrophils were incubated with 5 μ M CellROX Deep Red (C10422, ThermoFisher) at 37°C for 30 min in phenol red-free RPMI1640 supplemented with 2 mM glutamine, 100 U/mL Pen/Strep (11548876, ThermoFisher), 100 μ M sodium pyruvate, and 10 mM HEPES (12509079, ThermoFisher). Cells were washed with RPMI1640 and acquired as described above.

4.6 | Blood and Clinical Biochemistry

Cardiac blood was analyzed using a Veterinary Blood Cell Counter BC-5000 VET (Mindray). Plasma analysis was performed using an MNCHIP PointCare V3 analyzer (RAL SA).

4.7 | Tissue Processing, Embedding, and Staining

Tissues were fixed in 10% formalin, processed and embedded in paraffin or incubated in 30% sucrose overnight before embedding in OCT-Tissue-Tek medium (4583, Sakura). For macrophage and UCP1 protein visualization, paraffin sections were stained with rabbit anti-F4/80 (1:750) or rabbit anti-UCP1, followed by anti-rabbit HRP. Antigen–antibody–HRP complexes were visualized with 3-3'-diaminobenzidine. For the visualization of intratissue lipid droplets, cryosections were incubated in a 0.416% (w/v) Oil Red O solution (O0625, Sigma-Aldrich), washed, and counterstained with hematoxylin. Brightfield images were acquired using a NanoZoomer-2.0 HT C9600 digital scanner (Hamamatsu) with a 20X objective.

4.8 | RNA Extraction and cDNA Synthesis

For experiments at IRB Barcelona, tissues were homogenized in TRIzol (15596018, ThermoFisher) using a Precellys 24 Homogenizer (Bertin). RNA obtained by chloroform-induced separation was purified using the RNeasy Mini Kit (74106, Qiagen) following the manufacturer's protocol. cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (10186954, Applied Biosystems) as per the manufacturer's instructions. For experiments at McGill University, tissues were homogenized in QIAzol (79306, Qiagen) buffer, and RNA was purified using the RNeasy Mini Kit (Qiagen), following the manufacturer's protocol. cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

4.9 | Quantitative PCR (qPCR)

For experiments at IRB Barcelona, GoTaq qPCR Systems (A6002, Promega) were used as the master mix for determining relative gene expression. *Actb* was used as an internal control for normalization using the $2^{-\Delta\Delta C_t}$ method. All primer sequences are shown in Table S1. For experiments at McGill University, TaqMan Gene Expression Assays were used for determining relative gene expression of *Serpine1*, *Apl1l* and *Adgre1* (Mm00802529_m1). TATA-box binding protein (*Tbp*) was used as the internal control, and relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method.

4.10 | Bulk mRNA Sequencing

Bone marrow neutrophils were prepurified using magnetic microbeads as stated above. Neutrophils (DAPI⁻ CD11b^{hi} GR1^{hi}) were isolated using fluorescence-activated cell sorting and cell pellets frozen at -80°C until RNA extraction. Total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The concentration of total RNA extractions was quantified with the Nanodrop One (ThermoFisher), and RNA integrity was assessed with the RNA ScreenTape assay of the TapeStation 4200 platform (Agilent). Libraries for RNA-Seq were prepared at the IRB Barcelona Advanced Genomic Technologies core facility. Briefly, mRNA was isolated from 200 ng of total RNA and used to generate dual-indexed cDNA libraries with the Illumina stranded mRNA ligation kit (Illumina) and UD Indexes Set A (Illumina). Thirteen cycles of PCR amplification were applied to all libraries. Libraries were quantified using the Qubit dsDNA HS assay (Invitrogen) and quality controlled with the TapeStation D5000 assay (Agilent). An equimolar pool was prepared with the eleven libraries and submitted for paired-end 150 nt sequencing on a NovaSeq6000 S4 (Illumina). More than 191 Gbp of reads were produced, with a minimum of 52 million paired-end reads per sample.

4.11 | Bioinformatic Analysis

Analysis of bulk RNA-seq data was performed in R (v4.1.3 & v4.6.0) using the Bioconductor package. Reads were aligned with STAR (v2.7.10a) [50]. Differential expression analysis was

performed using Limma (v3.64.3) [51]. A gene was considered differentially expressed when both an empirical Bayes moderated *F*-test and at least one pairwise moderated *t*-test passed a Benjamini-Hochberg corrected *q*-value < 0.05 and exhibited a $\text{Log}_2\text{FoldChange} \geq 1$ in that contrast.

Normalized gene expression data of *SERPINE1* in human tissues was acquired through the online portal of the CZ CELLxGENE Discover platform by filtering for *SERPINE1* as the gene of interest in the corresponding human tissues [39]. Gene expression data of aggregated cell types were manually curated to yield a final list of definitive specific cell types.

4.12 | Statistical Analysis

Data were analyzed using GraphPad Prism (version 10) as indicated in figure legends. Briefly, comparison of two groups was performed using a two-tailed unpaired *t*-test, comparison of three or four groups was performed using one-way ANOVA, and comparison of longitudinal data was performed using two-way ANOVA. Data are presented as mean $\pm$ standard error of the mean. All experiments were repeated at least twice, and data were pooled. All data points represent biological replicates. Differences are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Author Contributions

S.F.: conceptualization, investigation, analysis, figure preparation, help with writing. F.S.: conceptualization, investigation, analysis, help with writing. L.S.: investigation, analysis. B.M.C. and Y.M.G.: investigation and analysis. C.D., R.A.S.A., and N.S.C.: methodology, writing. M.T.K. and S.K.W.: conceptualization, funding, project administration, supervision, visualization, writing and editing.

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Ethics Approval Statement for Human and/or Animal Studies

All mouse experiments at IRB Barcelona were approved by the Animal Experimentation Ethics Committee of Parc Científic de Barcelona and the Generalitat de Catalunya Animal Care and Use Committee (protocol CEEA-PCB-23-091). All experiments performed at McGill University were approved by the Animal Care Committee of McGill University (protocol MCGL-5188, May 3, 2023).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are contained in the manuscript or available from the corresponding author upon reasonable request. Gene expression data generated by bulk mRNA sequencing are available in the Gene Expression Omnibus under the accession code GSE341717.

Permission to Reproduce Material from Other Sources

Figure 1F was created in BioRender under agreement NK29YR5AU8. Created in BioRender. Wculek, S. (2026) <https://BioRender.com/5ry5sqq>.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting File: eji70271-sup-0001-SuppMat.pdf.