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Adipose tissue knockdown of lysozyme reduces local inflammation and improves adipogenesis in high-fat diet-fed mice

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Abbreviations: AT: Adipose tissue; HFD: High-fat diet; HOMA-IR: homeostasis model assessment-insulin resistance index; iWAT: Inguinal white adipose tissue; KD: knockdown; LBP: Lipopolysaccharide-binding protein; LPS: Lipopolysaccharide; LYZ: human lysozyme gene; Lyz2: Mouse lysozyme gene; NC: Negative control; ND: Normal diet; PBS: phosphate-buffered saline; pgWAT: Perigonadal white adipose tissue; SAT: Subcutaneous adipose tissue; SVF: Stromal vascular cell fraction; T2D: Type 2 diabetes; TLR4: Toll-like receptor 4; VAT:Visceral adipose tissue.

Abstract

Chronic systemic low-level inflammation in metabolic disease is known to affect adipose tissue biology. Lysozyme (LYZ) is a major innate immune protein but its role in adipose tissue has not been investigated. Here, we aimed to investigate LYZ in human and rodents fat depots, and its possible role in obesity-associated adipose tissue dysfunction. *LYZ* mRNA and protein were identified to be highly expressed in adipose tissue from subjects with obesity and linked to systemic chronic-low grade inflammation, adipose tissue inflammation and metabolic disturbances, including hyperglycemia, dyslipidemia and decreased markers of adipose tissue adipogenesis. These findings were confirmed in experimental models after a high-fat diet in mice and rats and also in o*b/ob* mice. Importantly, specific inguinal and perigonadal white adipose tissue lysozyme (*Lyz2*) gene knockdown in high-fat diet-fed mice resulted in improved adipose tissue inflammation in parallel to reduced lysozyme activity. Of note, *Lyz2* gene knockdown restored adipogenesis and reduced weight gain in this model. In conclusion, altogether these observations point to lysozyme as a new actor in obesity-associated adipose tissue dysfunction. The therapeutic targeting of lysozyme production might contribute to improve adipose tissue metabolic homeostasis.

Keywords: Adipogenesis, adipose tissue, gene knockdown, lysozyme, obesity.

Introduction

The existence of a positive and persistent increased energy balance in obesity is associated with a chronic low-grade inflammatory state in which adipose tissue (AT) produces increased amounts of proinflammatory adipokines resulting in peripheral insulin resistance and dyslipidemia [1–3]. Obesity-related inflammatory activity is characterized by dynamic changes in the composition and function of immune cells in insulin-dependent tissues (adipose tissue, liver and muscle), with well-known consequences in these tissues [4]. Obesity-associated adipose tissue inflammation is characterized by increased inflammatory M1 macrophage accumulation that surrounds adipocytes in “crown-like structures” and produces considerable amounts of proinflammatory cytokines [5,6]. Furthermore, infiltration of other innate immune cells, such as mast cells and neutrophils, has also been found in inflamed adipose tissue from

obese humans and mice [7–9]. One of the potential triggers of chronic inflammation has been attributed to increased gut permeability and translocation into bloodstream, with different microbial components (lipopolysaccharide) detectable in the circulation, impacting systemic inflammation and the physiology of different organs, being adipose tissue the most studied [10,11]. In fact, the depletion of pattern recognition receptors sensing microbial products [CD14, lipopolysaccharide-binding protein (LBP) and Toll-like receptor 4 (TLR4)] led to attenuated effects on metabolism and adipose tissue inflammation [10,12–14].

Lysozyme (LYZ, muramidase or N-acetylmuramylhydrolase) is an antimicrobial protein found in granules of neutrophils and macrophages [15] and in a wide diversity of biological fluids [16]. This enzyme has primarily bacteriolytic function, able to disrupt bacterial cell wall peptidoglycan (hydrolysis of β1-4 linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues) in Gram-positive and Gram-negative bacteria [16,17]. Together with other antimicrobial proteins, lysozyme is an important immune effector that carries out immunomodulatory activities, which trigger a proper response of innate immune system [18].

Increased plasma lysozyme levels in obesity has recently been associated with hyperglycemia, insulin resistance, dyslipemia and inflammatory parameters [19]. Interestingly, this study also demonstrated that lysozyme was expressed in human subcutaneous and visceral adipose tissue [19]. In fact, a previous transcriptomic study revealed that two years bariatric surgery-induced weight loss led to decreased SAT *LYZ* mRNA levels [20], suggesting a relationship between adipose tissue lysozyme and obesity. However, to the best of our knowledge, whether adipose tissue lysozyme levels are associated to obesity-associated metabolic disturbances or the impact of lysozyme on adipose tissue remain to be investigated. Since some studies [21–25] supported the importance of antimicrobial proteins and microbial-derived products signalling on adipose tissue physiology, here, we aimed to investigate LYZ in human and rodents fat depots, and its possible role in obesity-associated adipose tissue dysfunction.

Materials and Methods

Human study

Participants recruitment. A group of 326 [131 visceral (VAT) and 195 subcutaneous (SAT)] adipose tissues from participants with normal body weight and different degrees of obesity (with BMI within 20 and 68 kg/m^2) were analysed. These subjects were recruited at the Endocrinology Service of the Hospital de Girona "Dr Josep Trueta". All subjects were of Caucasian origin and reported that their body weight had been stable for at least three months before the study. Subjects were studied in the post-absorptive state. They had no systemic disease other than insulin resistance, T2D and/or obesity and all were free of any infections in the previous month before the study. Liver diseases (specifically tumoral disease and HCV infection) and thyroid dysfunction were excluded by biochemical work-up. Inclusion/exclusion of subjects taking drugs did not significantly alter the results. All subjects gave written informed consent, validated and approved by the ethical committee of the Hospital of Girona "Dr Josep Trueta", after they were explained the purpose of the study. Samples and data from patients included in this study were provided by the FATBANK platform promoted by the CIBEROBN and coordinated by the IDIBGI Biobank (Biobanc IDIBGI, B.0000872), integrated in the Spanish National Biobanks Network and they were processed following standard operating procedures with the appropriate approval of the Ethics, External Scientific and FATBANK Internal Scientific Committees.

Adipose tissue handling. AT samples were obtained from SAT and VAT depots during elective surgical procedures (cholecystectomy, surgery of abdominal hernia and gastric by-pass surgery). Tissue handling was carried out under strictly aseptic conditions and immediately flash-frozen in liquid nitrogen before being stored at -80 ºC. The isolation of adipocyte and stromal vascular fraction cells (SVF) was performed from 5 SAT and 5 VAT adipose tissue samples. These samples were washed with phosphate-buffered saline (PBS) and suspended in an equal volume of PBS supplemented with 1% penicillin-streptomicin and 0.1% collagenase type I prewarmed to 37 ºC. The tissue was placed in shaking water bath at 37 ºC with continuous agitation for 60

min and centrifuged for 5 min at 300-500 *g* at room temperature. The mature adipocytes containing supernatant was recollected. The pellet was idenfied as the SVF. $CD14^+$ cell fraction was isolated from SVF using magnetic immunobeads technology using a column and magnetic separator, according to the manufacturer's instructions (Miltenyi Biotec S.L., Madrid, Spain). Isolated mature adipocytes and SVF were stored at -80 ºC for gene expression analysis.

Analytical methods. Serum glucose concentration, glycated hemoglobin (HbA1c), serum insulin, homeostasis model assessment-insulin resistance index (HOMA-IR), total-, HDL- and LDL-cholesterol and fasting triglycerides were measured as previously described [26].

Animal experiments

Effects of HFD-induced weight gain in rats. Twenty male Wistar rats (four-week old, breeding house of the University of Navarra) were housed in individual cages and fed *ad libitum* during an average of 6 months either a normal chow diet [ND (12.1 kJ/g: 4% fat, 48% carbohydrate and 14% protein); n=10] or a high-fat diet [HFD (23.0 kJ/g: 60% fat, 27% carbohydrate and 14% protein); n=10]. Body weight and food intake were recorded to monitor progression of diet-induced obesity. After an overnight fast, rats were sacrificed by decapitation and perigonadal white adipose tissue (pgWAT) was collected and frozen in liquid nitrogen. All experimental procedures conformed to the European Guidelines for the Care and Use of Laboratory Animals (directive 2010/63/EU) and were approved by the Ethics Committee for Animal Experimentation of the University of Navarra (049/10).

Effects of genetic obesity in mice. Ten-week-old male and female C57BL/6J wild-type (n=20) and genetically obese *ob/ob* (n=19) mice (Harlan Laboratories Inc., Barcelona, Spain) were housed in specific genotype groups (n=3-4 per cage), in a room with controlled temperature (22 ± 2 °C), and a 12:12 light-dark cycle (lights on at 08:00 am). They were fed standard laboratory chow (Diet 2014S, Teklad Global Diets, Harlan, Barcelona, Spain) with water *ad libitum* during 22 weeks.

Effects of HFD-induced weight gain in mice. Ten-week-old male and female C57BL/6J wild-type were fed standard laboratory chow (n=10) or high-fat diet (HFD, F3282, BioServe) (n=12) with water *ad libitum* during 22 weeks, and housed in specific diet groups (n=3-4 per cage). In both *ob/ob* and HFD experiments, animals were sacrificed after 12 h fasting by CO_2 inhalation. perigonadal (pg) and inguinal (i) white adipose tissue (WAT) were rapidly dissected out, weighed, frozen in liquid nitrogen, and stored at -80 °C until RNA extraction. The research was conducted in accordance with the European Guidelines for the Care and Use of Laboratory Animals (directive 2010/63/EU) and was approved by the Ethical Committee for Animal Experimentation of the University of Navarra (041/08), as previously described[27].

Effects of short-term specific iWAT and pgWAT Lyz2 gene knockdown.

Eight-week-old male C57BL/6J wild-type were housed in specific treatment groups (shC or shLyz2, n=2-3 per cage) and fed a high-fat diet (HFD, TD.08811, ENVIGO) (n=20) with water *ad libitum* during 3 weeks. Then, lentiviral injection in iWAT and pgWAT was performed at week 11. To inject lentiviral particles in both iWAT and pgWAT, mice were anesthetized by isoflurane before dissection of the skin. The lentiviral preparation ($1 \times 10^{7–8}$ plaque-forming units in a volume of 100 µl) was injected into the right and left pgWAT and iWAT depot, which were distributed first in 6 injections of 10 µl for each pgWAT, and then 4 injections of 8 µl for each iWAT (as showed below in Figure 4A-B). Each mouse was injected with ~185 µl of lentiviral preparation. Mice were randomly allocated to the treatment groups (shC group versus sh*Lyz2* group, n=4-6 mice/group). Insulin and glucose tolerance test were performed at week 18. For glucose tolerance tests, glucose in aqueous solution was administered intraperitoneally (2.5 g glucose/kg) to overnight-starved mice, and glycaemia in blood obtained from the tail was measured 15, 30, 60, 90, 120 and 150 minutes after glucose injection. For insulin tolerance tests, insulin (Actrapid; Novo Nordisk Pharma A/S, Bagsvaerd, Denmark) in saline solution was administered intraperitoneally (0.75 UI/kg) to mice, and glycaemia in blood obtained from the tail was measured 15, 30, 45, 60 and 90 minutes after glucose injection. At week 15 (n=8) and 19 (n=12), 4 and 8 weeks after lentiviral injection, mice were sacrificed by CO_2 inhalation.

Then, pgWAT and iWAT were rapidly dissected out, frozen in liquid nitrogen, and stored at -80 °C until RNA/protein extraction. To evaluate the efficiency of lentiviral knockdown, three independent biopsies from gWAT and two from iWAT were analysed in each mice. Lysozyme activity was measured using EnzChek Lysozyme Assay Kit (E-22013,Life Technologies SA, Spain) following manufacturer's procedure. The research was conducted in accordance with the European Guidelines for the Care and Use of Laboratory Animals (directive 2010/63/EU) and was approved by the Ethical Committee for Animal Experimentation of Barcelona Science Park (PCB).

Lentiviral shRNA-Lyz2 particles production

Four different short-hairpin-*Lyz2* (clone set against mouse *Lyz2*, NM_017372.3) primer sequences and random negative control (NC) sequence that did not have targets for any gene were synthesized by Tebu-bio (Tebu-bio, Spain, SL). Lentivirus-targeted *Lyz2* was obtained by cotransfection of shRNA plasmids against *Lyz2* and a combination of packaging and envelope plasmid from Addgene (pCMV-VSV-G and pCMV-dR8.2 dvpr) into HEK293T using LipoD293 transfection reagent following manufacturers' instructions.

Gene expression analysis

Total RNA was extracted and purified form human and rat AT using RNeasy Lipid Tissue Mini kit (QIAGEN, Izasa SA, Barcelona, Spain) and integrity was checked by Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA). Total RNA was quantified by means of a spectrophotometer (GeneQuant, GE Health Care, Piscataway, NJ). The same amount of total RNA was reverse transcribed to cDNA from all samples using High Capacity cDNA Archive kit (Applied Biosystems, Darmstadt, Germany) following manufacturers' instructions. Gene expression was assessed by real time PCR using LightCycler 480 Real-Time PCR System (Roche Diagnostics SL, Barcelona, Spain), using TaqMan technology suitable for relative gene expression quantification. Commercially available and pre-validated TaqMan primer/probe sets were used for gene expression determinations (Applied Biosystems). A threshold cycle (Ct value) was obtained for each amplification curve. ΔCt value was first calculcated by

substracting the Ct value for corresponding endogenous control (human cyclophilin A, *PPIA*) in each sample. Fold changes compared with the endogenous control were then determined by calculating $2^{-\Delta Ct}$, so gene expression results are expressed as expression ratio relative to preselected and validated endogenous controls. Replicates and positive and negative controls were included.

Human primer/probe sets used were: peptidylprolyl isomerase A (cyclophilin A) (4333763, *PPIA* as endogenous control), lysozyme (*LYZ*, Hs00426232_m1), fatty acid synthase (*FASN*, Hs01005622_m1), acetyl-CoA carboxylase alpha (*ACACA*, Hs00167385_m1), solute carrier family 2 member 4 (*SLC2A4*, Hs00168966_m1), peroxisome proliferator activated receptor gamma (*PPARG*, Hs01115513_m1), fatty acid binding protein 4 (*FABP4*, Hs01086177_m1), insulin receptor substrate 1 (*IRS1*, Hs00178563_m1), adiponectin (*ADIPOQ*, Hs00605917_m1), lipopolysaccharide binding protein (*LBP*, Hs00188074_m1) leptin (*LEP*, Hs00174877_m1), interleukin 6 (*IL6*, Hs00985639_m1), interleukin 8 (*IL8*, Hs00174103_m1), tumor necrosis factor alpha (*TNF*, Hs00174128_m1), integrin subunit alpha X (*ITGAX*, Hs00174217_m1), heme oxygenase 1 (*HMOX1*, Hs0157965_m1), CD68 molecule (*CD68*, Hs00154355_m1), mannose receptor, C type 1 (*MRC1* or *CD206*, Hs00267207_m1).

Rat primer/probe sets used were: Lysozyme (*Lyz2*, Rn00562794_m1), adiponectin (*Adipoq*, Rn00595250_m1), interleukin 6 (*Il6*, Rn01410330_m1), heme oxygenase 1 (*Hmox1*, Rn00561387_m1).

Mouse primer/probe sets used were: peptidylprolyl isomerase A (*Ppia*, Mm02342430_g1), EUK18S rRNA (*18S*, 4352655), lysozyme (*Lyz2*, Mm01612741_m1), adiponectin (*Adipoq*, Mm00456425_m1), fatty acid binding protein 4 adipocyte (*Fabp4*, Mm00445880_m1), fatty acid synthase (*Fasn*, Mm00662319_m1), solute carrier family 2 member 4 (*Slc2a4*, Mm00436615_m1), perilipin 1 (*Plin1*, Mm00558672_m1), leptin (*Lep*, Mm00434759_m1), peroxisome proliferator activated receptor gamma (*Pparg*, Mm00440940_m1), interleukin 6 (*Il6*, Mm00446190_m1), tumor necrosis factor (*Tnf*, Mm00443258_m1), chemokine (C-C motif) receptor 2 (*Ccr2*, Mm04207877_m1) and integrin alpha X (*Itgax*, Mm00498701_m1).

Protein analysis

Adipose tissue proteins were extracted directly in radioimmnuno precipitation assay (RIPA) buffer (0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 150 mmol/L NaCl, and 50 mmol/L Tris-HCl, pH 8.0), supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride). Cellular debris and lipids were eliminated by centrifugation of the solubilized samples at 12,000 *g* for 10 min at 4 ºC, recovering the soluble fraction. Protein concentration was determined using the RC/DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). RIPA protein extracts (15 μg) were run in 12.5% SDS-PAGE and transferred to nitrocellulose membrane by conventional procedures. After blocking with 5% BSA in TBS-Tween, membranes were incubated overnight with purified rabbit anti-human LYZ monoclonal antibody (1/1000) (ab36362; Abcam, Cambridge, UK) followed by incubation with horseradish peroxidase-conjugated polyclonal rabbit anti-mouse antibody. Protein signal was detected by enhanced chemiluminescence kit (GE Healthcare, Madrid, Spain). Ponceau staining was performed as protein loading control.

Statistical analyses

Statistical analyses were performed using the SPSS 21.0 software. Unless otherwise stated, descriptive results of continuous variables are expressed as mean and SD for Gaussian variables or median and interquartile range for non-Gaussian variables. Before statistical analysis, normal distribution and homogeneity of the variances were evaluated using Levene's test. Parameters that did not fulfil normal distribution were mathematically transformed to improve symmetry for subsequent analyses. The relation between variables was analyzed by simple correlation (using Spearman's tests). One-way ANOVA for multiple comparisons (using *post-hoc* by Bonferroni's) and unpaired t-tests were used to compare clinical variables and *LYZ/Lyz2* gene expression relative to obesity. In animal experiments, data are expressed as means (SEM). Levels of statistical significance were set at $p<0.05$.

Results

Adipose tissue lysozyme in obesity

Anthropometrical and clinical characteristics are shown in Table 1. Human *LYZ* mRNA and protein levels were highly detectable in adipose tissue (AT) (Figure 1A-C). In both subcutaneous (SAT) and visceral (VAT), *LYZ* was significantly much more expressed than other antimicrobial proteins known to be present in human AT (such as *LBP*, *LTF*, *BPI*, *SPD*, *CD14*, *S100A9* and *S100A8*) and even more than some adipogenic genes, such as *PPARG*, *ACACA*, *SLC2A4* and *IRS1* (Figure 1A-B). *LYZ* expression was similar to other known highly expressed AT proteins, such as *FASN* and *LEP* (Figure 1A-B). Adipose tissue *LYZ* gene expression were significantly increased in subjects with obesity ($p<0.0001$, Figure 1D-E, Table 1). Adipose tissue *LYZ* mRNA were associated with LYZ protein levels (Figure 1C). Although *LYZ* was expressed in adipocytes, its main expression was detected in cells from the stromal vascular fraction in both VAT and SAT (Figure 1F), and specifically in $CD14^+$ cells (Figure 1G). SAT *LYZ* correlated with VAT *LYZ* ($r=0.54$, $p<0.0001$). In both SAT and VAT, *LYZ* mRNA was increased concurrently with BMI ($r=0.56$, $p<0.0001$ in SAT and $r=0.37$, $p<0.0001$ in VAT) and fat mass ($r=0.50$, $p<0.0001$ in SAT and $r=0.28$, $p=0.001$ in VAT).

LYZ as a tissue inflammatory biomarker

LYZ mRNA was significantly and positively associated with the expression of AT inflammatory markers such as *TNF*, *ITGAX* or *HMOX1* in both VAT and SAT (Figure 2). *LYZ* gene expression negatively correlated with *CD206*/*CD68* ratio [a specific and indirect marker of M2 macrophages [28]] in VAT, but not with *CD68*, a non-specific macrophage marker (Figure 2). Most of the association related to inflammatory markers remained significant after controlling for BMI. In VAT, *TNF* ($\beta=0.41$, $p<0.001$), *ITGAX* ($\beta=0.82$, $p<0.0001$), and *HMOX1* ($\beta=0.79$, $p<0.0001$) contributed independently to *LYZ* gene expression variance after controlling for BMI. In SAT, *TNF* ($\beta=0.21$, $p=0.009$) and *HMOX1* ($\beta=0.21$, $p=0.046$) contributed independently to *LYZ* gene expression variance after controlling for BMI.

LYZ is associated with metabolic dysfunction

Importantly SAT *LYZ* positively correlated with obesity-associated metabolic disturbances such as fasting serum glucose, HOMA-IR and fasting triglycerides (Table 1) while negatively correlated with markers of adipogenesis (*FASN, ACACA, SLC2A4* and *IRS1)* (Figure 2).

Replication in mice and rats models

The findings in humans were further substantiated in animal models. In experimental models of genetic (*ob/ob*) and high-fat diet (HFD)-induced obesity, *Lyz2* mRNA was significantly increased in both inguinal (i) and perigonadal (pg) WAT (Figure 3A) and negatively correlated with *Adipoq*, *Slc2a4*, *Pparg*, *Fasn*, *Plin1* and *Fabp4* mRNA levels, mainly in pgWAT (Figure 3B). Both iWAT and pgWAT *Lyz2* mRNA were positively correlated with expression of proinflammatory (*Il6*, *Tnf*, *Itgax*) and obesogenic (*Lep*) genes, serum glucose and AUC-GTT, and only pgWAT *Lyz2* with body and fat weight (Figure 3B).

In rats, HFD-induced weight gain led to increased expression of *Lyz2* mRNA levels in pgWAT (141.5% increase, p=0.003) compared to normal diet (Figure 3C). pgWAT *Lyz2* mRNA levels were positively correlated with body weight, pgWAT weight, circulating leptin, HOMA-IR and pgWAT *Il6* and *Hmox1* mRNA, while negatively correlated with *Adipoq* mRNA levels (Figure 3D-J).

All these results showed a significant link between AT LYZ and obesity associated-adipose tissue dysfunction, suggesting that the downregulation in AT LYZ might improve AT adipogenesis. To investigate the impact of AT LYZ in obesity-associated adipose tissue physiology, specific iWAT and pgWAT *Lyz2* gene knockdown (KD) in HFD-fed mice was performed using lentiviral particles with shRNA against *Lyz2* mRNA.

Effects of specific iWAT and pgWAT Lyz2 knockdown on adipose tissue physiology

At 4 weeks of lentiviral injection, *Lyz2* gene expression and protein activity were significantly decreased in both iWAT and pgWAT (Figure 4A-B), especially in iWAT (p=0.005) compared with pgWAT (p=0.02). Of note, WAT *Lyz2* silencing impacted on adipose tissue inflammation

and adipogenesis (Figure 4C-F). Specifically, iWAT *Lyz2* gene KD resulted in decreased *Tnf*, *Itgax* and *Ccr2* mRNA levels (Figure 4C), and increased expression of adipogenic genes (*Adipoq, Fabp4, Fasn, Slc2a4, Plin1* and *Pparg*) (Figure 4D). In pgWAT, *Lyz2* gene KD also was associated with decreased expression of inflammatory-related genes (Figure 4E) and increased *Fasn* and *Plin1* mRNA levels (Figure 4F).

At 8 weeks of lentiviral injection, the decrease of *Lyz2* gene expression on iWAT was slightly attenuated, even though it remained statistically significant (Figure 4G). Consistently, iWAT *Lyz2* KD resulted in decreased *Tnf* and *Itgax* (Figure 4H) and increased *Fabp4, Fasn, Slc2a4* and *Plin1* (Figure 4I) mRNA levels. In contrast, the effects of lentiviral injection in pgWAT were lost at week 8 (Figure 4J). In fact, similar to proinflammatory genes (*Tnf* and *Ccr2*), pgWAT *Lyz2* mRNA tended to be increased, whereas *Plin1* mRNA decreased in shLyz2 mice (Figure 4J-L).

AT LYZ silencing attenuated body weight gain

Even though no significant changes in body weight were observed between shC- and shLyz2-treated mice (Figure 5A), body weight gain was significantly reduced in shLyz2-injected mice during the week period with the most WAT *Lyz2* gene knockdown (from week 0 to 4) (Figure 5B). At week 7 after lentiviral injection, blood glucose during GTT and ITT remained unchanged (Figure 5C-D), indicating that short-term specific knockdown of WAT *Lyz2* gene expression did not impact on systemic glucose metabolism. However, WAT *Lyz2* KD resulted in a non-significant (p=0.07) decreased blood glucose concentration at 15 min during ITT (Figure 5E). No significant differences on plasma lipopolysaccharide binding protein (LBP) were found (Figure 5F).

Discussion

To the best of our knowledge this is the first study investigating adipose tissue lysozyme linked to obesity and insulin resistance. AT *LYZ* mRNA levels were significantly increased in comparison to other antimicrobial proteins expressed in human adipose tissue [21,23,24,29,30]

and comparable to highly expressed adipose tissue proteins, such as *FASN* and *LEP*. AT *LYZ* mRNA levels were significantly associated with both obesity and obesity-associated metabolic disturbances, including insulin resistance and disturbed glucose metabolism, dyslipidemia and chronic-low level inflammation. AT *LYZ* mRNA was proportional to LYZ protein levels. Increased AT *Lyz* mRNA was confirmed in animal models of obesity, both after a high-fat diet and in genetic obesity. iWAT and pgWAT *Lyz2* gene KD prevented body weight gain and impacted on adipose tissue, decreasing local AT inflammatory activity and improving adipogenesis.

Antibacterial and immunomodulatory activity of lysozyme is mediated by its capacity to hydrolyse bacterial peptidoglycans [31]. Digestion of bacterial peptidoglycan by lysozyme is required in inflammatory/infectious processes to promote macrophage recruitment [32] and inflammasome activation [33,34]. Importantly, pattern recognition receptors activated downstream of lysozyme-mediated peptidoglycan degradation have been strongly associated with obesity-associated metabolic disturbances and adipose tissue dysfunction [35–41]. Chi et al demonstrated that bacterial peptidoglycan attenuated lipid storage capacity and increased lipolysis in adipose tissue and adipocytes [42].

Specific adipose tissue lysozyme knockdown in HFD-fed mice resulted in improved adipose tissue functionality, increasing expression of adipogenesis- and adipose tissue expandability-related genes in parallel to decreased markers of adipose tissue inflammation (*Tnf*) and classical M1 macrophages (*Itgax* and *Ccr2*). Of note, when the effects of gene knockdown decreased (at week 8), these improvements were slightly attenuated. The maximum effects of specific adipose tissue gene knockdown using lentiviral particles were at week 4. In line with this, a previous study with a similar gene knockdown procedure in adipose tissue, evaluated the effects of adipose gene knockdown at week 3 [43].

Current data suggest that the adipogenic effects observed in subcutaneous and visceral adipose tissue when lysozyme expression and activity were experimentally attenuated might be mediated by the reduction of high-fat diet-induced $CD11c^{+}$ macrophages recruitment in fat

depots [44,45]. Supporting this suggestion, lysozyme is an inducible marker of tissue macrophage activation [46,47] and its activity promotes macrophage recruitment [30,31]. In agreement with these experimental data, AT *LYZ* gene expression was positively correlated with proinflammatory cytokines (*TNF*, *IL6*) and macrophage markers (*ITGAX*, *HMOX1*) and negatively with CD206/CD68 ratio [a sign of macrophage M1 polarization [28]] and adipogenic gene expression in human adipose tissue. In addition, two years bariatric surgery-induced weight loss resulted in decreased SAT *LYZ* mRNA (-68.3%, p=0.002) in parallel to a significant amelioration in metabolic parameters and adipose tissue dysfunction markers (including increased expression of adipogenic and decreased inflammatory genes) [20].

Considering that both high-fat diet and genetic obesity promotes gut dysbiosis, increasing intestinal permeability and metabolic endotoxemia [10,48,49], the impact of gut dysbiosis on adipose tissue lysozyme cannot be excluded in these experiments. Otherwise, in lentivirus-induced adipose tissue *Lyz2* gene knockdown experiments, all mice were fed with high-fat diet and no difference on diet-induced gut dysbiosis would be expected in each experimental group (shC or shLyz2). In fact, no significant difference in the plasma LBP levels, previously reported as a metabolic endotoxemia marker [50,51], were observed. Further studies should be required to examine the impact of obesity-associated gut microbiota on adipose tissue lysozyme.

A limitation of current study was the lack of a longer term specific adipose tissue *Lyz2* gene knockdown. However, the widespread presence of macrophages in other tissues make quite challenging to perform a lysozyme conditional knock out selectively in the adipose tissue through Cre-loxP strategy. Another advantage of current experimental approach was that this is post-developmental.

Conclusions

Current findings point to lysozyme as a new actor in obesity-associated adipose tissue dysfunction. Modulation of adipose tissue lysozyme levels results in improved adipogenesis in the short term and might prevent diet-induced weight gain. However, the metabolic effects of

long term adipose *Lyz2* gene knockdown should be investigated in further experiments using improved gene knockdown strategies in fat depots.

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Table 1. Anthropometric and clinical parameters and VAT and SAT *LYZ* gene expression according to obesity in cohort 1. Bivariate correlations between VAT and SAT *LYZ* gene expression and clinical measurements in cohort 1.

VAT	**Non-obese**	**Non-obese+T2D**	**Obese**	**Obese+T2D**	**p[1]**	**VAT *LYZ* mRNA**	
						r	**p[2]**
N (men/women)	18/38	2/3	13/34	4/19	0.5		
Age (years)	49.5 ± 11.7	56.6 ± 14.5	42.4 ± 10.8[a,b]	44.7 ± 10.2	**0.003**	-0.23	**0.08**
BMI (kg/m^2)	24.8 ± 3.3	28.1 ± 1.5	44.4 ± 8.3[a,b]	44.1 ± 4.0[a,b]	**<0.001**	0.37	**<0.001**
Fat mass (%)	32.3 ± 6.8	37.0 ± 8.0	54.7 ± 10.9[a,b]	55.9 ± 8.3[a,b]	**<0.001**	0.28	**0.00 1**
Fasting glucose (mg/dl)	90.3 ± 10.5	172.4 ± 64.7[a]	92.9 ± 11.7[b]	133.0 ± 50.2[a,b,c]	**<0.001**	0.10	0.2
HOMA-IR	2.65 ± 2.27	3.04 ± 0.29	2.64 ± 1.28	6.32 ± 5.18	**0.04**	-0.04	0.8
Total cholesterol (mg/dl)	202.7 ± 41.4	234.8 ± 50.7	195.1 ± 35.0	181.0 ± 34.1[b]	**0.02**	-0.03	0.7
HDL-cholesterol (mg/dl)	58.76 ± 19.01	48.00 ± 9.95	58.61 ± 53.02	52.23 ± 12.35	0.8	-0.13	0.2
LDL-cholesterol (mg/dl)	121.81 ± 33.78	157.80 ± 43.67	118.93 ± 35.11	104.20 ± 33.34[b]	**0.02**	-0.08	0.4
Fasting triglycerides (mg/dl)[d]	93.5 (64.0-145.5)	171.0 (134.5-240.0)	97.0 (78.8-145.3)	138.0 (85.0-160.0)	0.08	0.17	0.05
hsCRP (mg/dl) [d]	0.18 (0.06-0.49)	0.60 (0.29-1.28)	1.10 (0.55-1.70)	0.68 (0.32-1.71)	**0.00 05**	0.36	**0.00 1**
LYZ mRNA (R.U.)[d]	0.14 (0.06-0.25)	0.19 (0.13-0.34)	0.25 (0.14-0.39)[a]	0.22 (0.11-0.33)	**0.00 2**	-	-
SAT	**Non-obese**	**Non-obese+T2D**	**Obese**	**Obese+T2D**	**p[1]**	**SAT *LYZ* mRNA**	
						r	**p[2]**
N (men/women)	16/78	2/4	17/50	4/24	0.4		
Age (years)	47.8 ± 9.2	55.2 ± 13.4	45.9 ± 10.5	45.6 ± 10.9	0.1	-0.13	0.07
BMI (kg/m^2)	25.1 ± 3.5	28.2 ± 1.4	42.9 ± 7.4[a,b]	44.2 ± 6.9[a,b]	**<0.001**	0.56	**<0.001**
Fat mass (%)	33.9 ± 6.3	37.5 ± 7.3	53.9 ± 10.7[a,b]	56.6 ± 10.4[a,b]	**<0.001**	0.50	**<0.001**
Fasting glucose (mmol/l)	89.3 ± 10.1	158.0 ± 67.8[a]	92.8 ± 11.9[b]	126.7 ± 47.1[a,b,c]	**<0.001**	0.21	**0.00 3**
HOMA-IR	2.22 ± 1.99	3.04 ± 0.29	2.57 ± 1.63	8.38 ± 8.08[a,c]	**0.00 1**	0.44	**1**
Total cholesterol (mg/dl)	193.9 ± 39.8	228.5 ± 47.9	190.5 ± 37.3	179.2 ± 31.2[b]	**0.03**	0.08	0.3
HDL-cholesterol (mg/dl)	63.44 ± 20.33	48.33 ± 8.94	59.69 ± 41.97	49.70 ± 10.26	0.1	-0.22	**0.00 2**
LDL-cholesterol (mg/dl)	114.21 ± 34.10	152.53 ± 41.13[a]	111.89 ± 32.32[b]	103.76 ± 28.90[b]	**0.02**	0.09	0.2
Fasting triglycerides (mg/dl)[d]	84.5 (59.8-105.3)	160.0 (105.5-220.0)[a]	93.0 (74.0-126.0)	138.0 (96.0-160.0)[a]	**<0.001**	0.33	**<0.001**
hsCRP (mg/dl) [d]	0.13 (0.04-0.33)	0.38 (0.12-0.98)	0.50 (0.24-1.10)	0.63 (0.33-1.94)	**0.02**	0.46	**<0.001**
LYZ mRNA (RU)[d]	0.16 (0.05-0.21)	0.23 (0.17-0.28)	0.34 (0.18-0.46)[a]	0.34 (0.23-0.47)[a]	**<0.001**	-	-

Unless otherwise indicated, the data are expressed as mean±SD.

One-factor ANOVA was used to derive p values (p^1). Bold values indicate statistical significance, which was set at $p<0.05$. p^2 indicated p value of spearman correlations.
[a] $p<0.05$ indicates significant differences compared to non-obese individuals (Bonferroni *post-hoc* test)
[b] $p<0.05$ indicates significant differences compared to non-obese+T2D individuals (Bonferroni *post-hoc* test)
[c] $p<0.05$ indicates significant differences compared to obese individuals (Bonferroni *post-hoc* test)
[d]Median (interquartile range)
BMI: body mass index, **HOMA-IR**: homeostasis model assessment-insulin resistance index, **RU:** relative units of gene expression, **SAT:** subcutaneous adipose tissue, **VAT:** visceral adipose tissue.

Figure legends

Figure 1

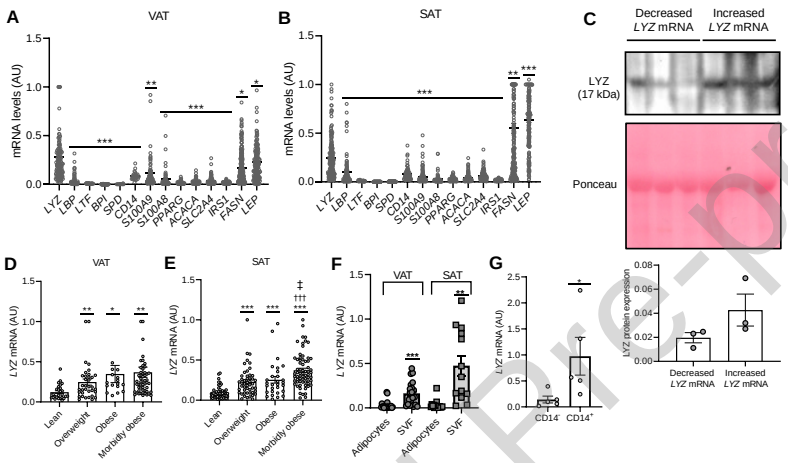


Figure 1. A-B) *LYZ, LBP, LTF. BPI, SPD, CD14, S100A9, S100A8, PPARG, ACACA, SLC2A4, IRS1, FASN* and *LEP* mRNA levels in VAT **(A)** and SAT **(B)** in all participants (n=131 in VAT and n=195 in SAT). *p<0.05,**p<0.01 and ***p<0.001 compared to *LYZ* mRNA. **C)** VAT LYZ protein in relation to *LYZ* mRNA levels in obese participants (n=6). **D-E)** *LYZ* mRNA in VAT **(D)** and SAT **(E)** according to obesity, being the number of participants as follows: lean (N=26), overweight (N=35), obese (N=17) and mordidly obese (N=53) in VAT, and lean (N=51), overweight (N=49), obese (N=29) and mordidly obese (N=66) in SAT. *p<0.05,**p<0.01 and ***p<0.001 compared to lean participants. †††p<0.001 compared to overweight participants. ‡p<0.05 compared to obese participants. **F-G)** *LYZ* mRNA in cells from stroma-vascular fraction (SVF) and adipocytes isolated from SAT (n=13) and VAT (n=24) **(F)** and in $CD14^-$ and $CD14^+$ cell fractions (n=5) **(G)**. *p<0.05 compared to $CD14^-$ cell fraction and **p<0.01 and ***p<0.001 compared to adipocytes.

Figure 2

Figure 2) Correlation chart showing bivariate correlations among *LYZ* and adipose tissue adipogenic (*PPARG*, *FASN*, *ACACA*, *GLUT4*, *IRS1*)-, obesity (*LEP*, *LBP*)- and inflammation (*TNF*, *ITGAX*, *HMOX1*, *CD206*, *CD68*)-related gene expression.*p<0.05; **p<0.01; ***p<0.001. Blue indicates negative and red positive correlations.

Figure 3

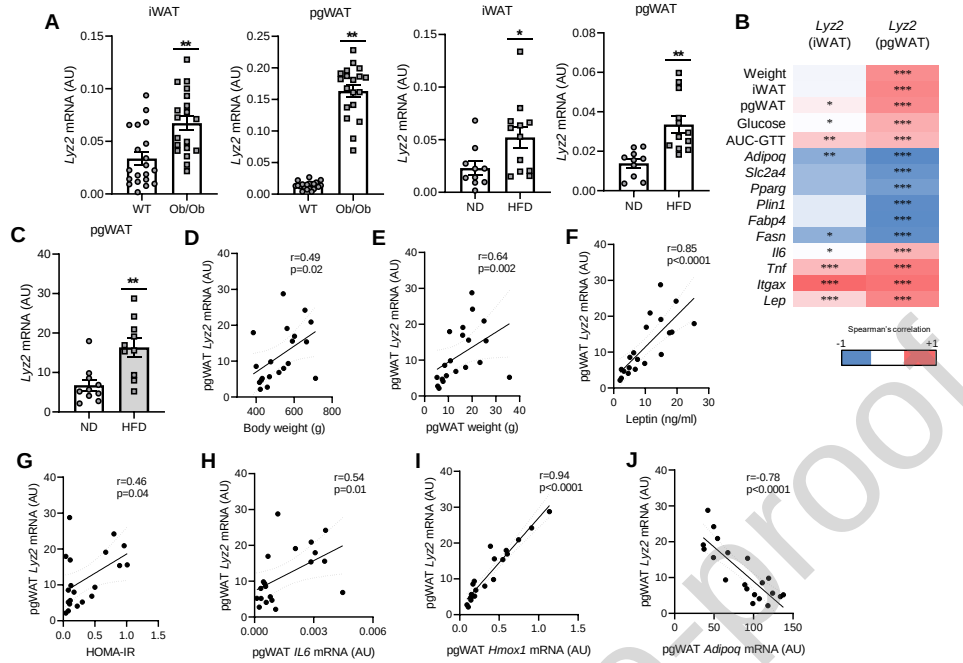


Figure 3. A) Effects of genetic (ob/ob)- and HFD -induced obesity on iWAT and pgWAT *Lyz2* mRNA in mice. The number of mice used in these experiments were as follows: 19 in ob/ob *vs* 20 in WT mice, and 12 in HFD- *vs* 10 in ND-fed WT mice. *p<0.05 and **p<0.01 compared to WT or ND-fed mice. **B)** Correlation chart showing bivariate correlations among iWAT and pgWAT *Lyz2* and weight, glucose, AUC-GTT and expression of adipogenic (*Adipoq*, *Slc2a4*, *Pparg*, *Plin1*, *Fabp4*, *Fasn*), inflammatory (*Il6*, *Tnf*, *Itgax*) and obesogenic (*Lep*) genes in all mice. Blue indicates negative and red positive correlations. *p<0.05; **p<0.01; ***p<0.001. **C)** Effect of HFD-induced obesity on pgWAT *Lyz2* mRNA in rats (n=10 in each group). **p<0.01 compared to ND-fed rats. **D-J)** Bivariate correlations between pgWAT *Lyz2* mRNA levels and body weight **(D)**, pgWAT weight **(E)**, leptin **(F)**, HOMA-IR **(G)**, and pgWAT *Il6* **(H)**, *Hmox1* **(I)** and *Adipoq* **(J)** mRNA levels in all rats.

Figure 4

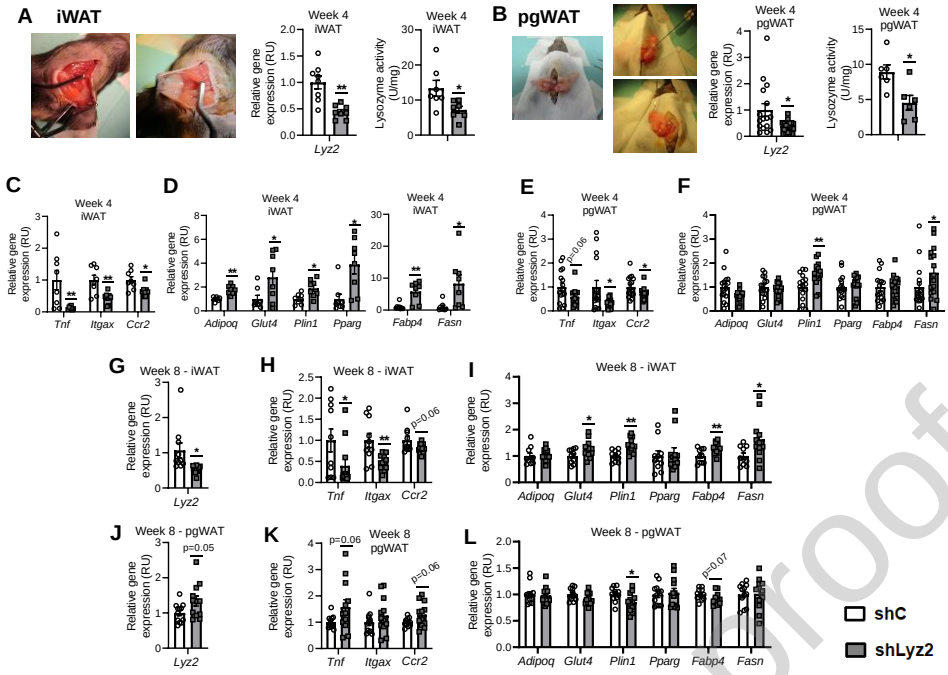


Figure 4. A-B) Methodological approach of the specific adipose tissue *Lyz2* gene knockdown procedure. Injection of lentiviral particles in both right and left iWAT **(A)** and pgWAT **(B)** and its impact on *LYZ* mRNA levels and protein activity. **C-L)** Effects of specific iWAT and pgWAT *Lyz2* gene knockdown on markers of adipose tissue expandability, adipogenesis and inflammation at week 4 **(C-F)** and 8 **(G-L)**.*p<0.05 and **p<0.01 compared to shC. Gene expression was analysed in 8 (week 4) and 12 (week 8) iWAT and 16 (week 4) and 12 (week 8) pgWAT samples, and LYZ protein activity in 7 iWAT and 6 pgWAT samples.

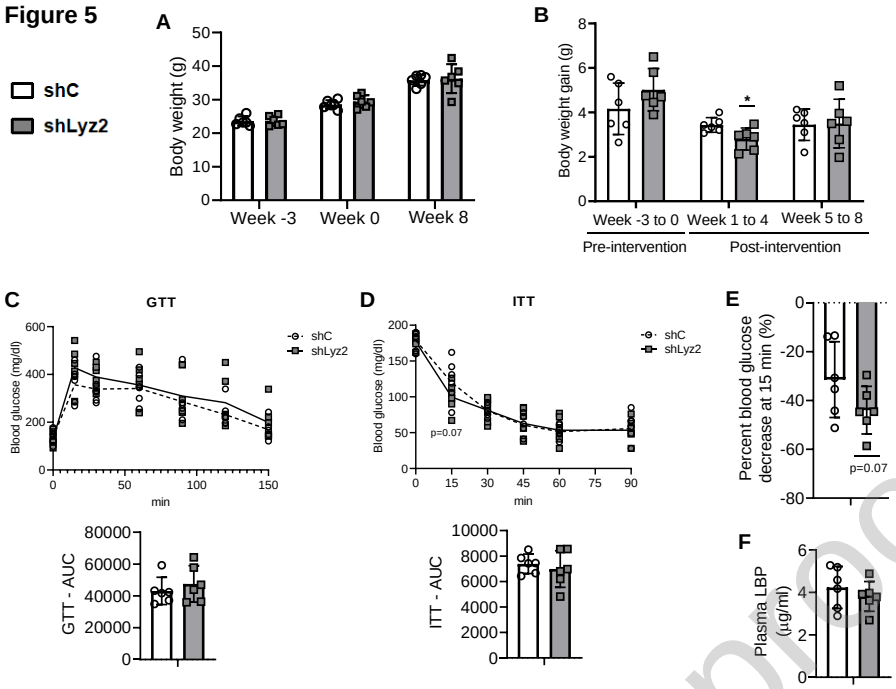
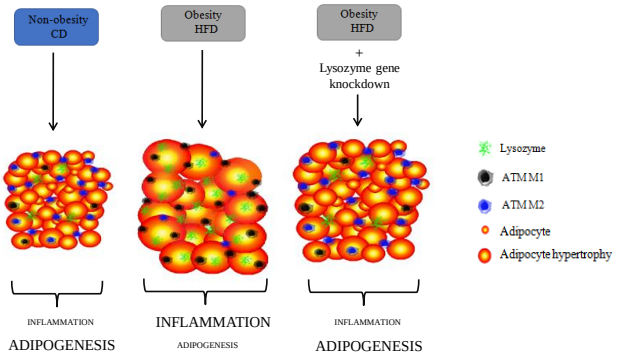


Figure 5. A-D) Effects of specific iWAT and pgWAT *Lyz2* gene knockdown (n=6 in each group) on body weight **(A)**, body weight gain according to lentiviral intervention period and gene knockdown efficiency **(B)**, GTT **(C),** ITT **(D),** percent change in blood glucose at 15 min during ITT **(E)** and plasma LBP levels **(F)**. *p<0.05 compared to shC.

Graphical abstract



Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights

- Lysozyme gene is highly expressed in human and mice subcutaneous and visceral adipose tissue in association to obesity.

- Adipose tissue lysozyme gene expression is linked to adipose tissue inflammation and obesity-associated metabolic disturbances.

- Specific adipose tissue lysozyme gene knockdown reduces adipose tissue inflammation and restores adipogenesis in high-fat diet-fed mice.