RESEARCH LETTER

Impact of weight loss on the expression of negative regulators of nuclear factor κB (*NLRP12*, *NLRC3*, and *NLRX1*) in subcutaneous adipose tissue

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Introduction Insulin resistance can be defined as a state of impaired response to insulin in insulin-targeted tissues. Insulin resistance is associated with obesity, type 2 diabetes, atherosclerosis, cardiovascular disease (CVD), metabolic syndrome, nonalcoholic fatty liver disease, and polycystic ovary syndrome.[1,2] Adipose tissue (AT) is an important factor in the pathogenesis of insulin resistance, as disturbances in its function contribute to development of low-grade chronic inflammation, which impairs insulin sensitivity. AT contains various immune cells, which regulate metabolic processes and immune responses of adaptive and innate immune system. The innate immune system recognizes a pathogen and aims to eliminate it, while causing the least possible damage to the host organism. A key component of the innate immune system is inflammation, which can be either of short duration (acute) or persist for a long time (chronic). The nuclear factor κB (NFκB) pathway plays a main role in the regulation of inflammation. Dysregulation in inflammation onset can result in low-grade chronic inflammation, which is one of the main contributors to development of insulin resistance. The function of the innate immune system response depends on pattern recognition receptors (PRRs), which identify pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). PRRs can be divided into 5 groups, including nucleotide-binding oligomerization domain-like receptors (NLRs).[3] The primary role of NLRs is to detect cytosolic PAMPs and DAMPs, inducing and regulating downstream signaling cascades that result in an inflammatory response. Several members of the NLR family, including *NLRC3*, *NLRP12*, and *NLRX1*, function as suppressors of excessive proinflammatory signaling through the NFκB pathway, whether it is the canonical or noncanonical pathway.[4] These NLRs bind to signaling molecules of the NFκB pathway, inhibiting its activity, reducing the production of proinflammatory cytokines, and preventing the activation of downstream signaling pathways. *NLRC3* is widely expressed throughout the body, but it is predominantly found in various immune system tissues.[5] It has been shown to inhibit the activity of several key pathways, including the NFκB, STING, and PI3K-mTOR ones.[6,7] *NLRP12* is expressed in various immune cells, including monocytes, dendritic cells, and granulocytes.[8] *NLRP12* can function both as an inflammasome and a negative regulator of inflammation.[9] *NLRX1* is expressed ubiquitously in various types of tissues. Its primary function is to negatively regulate antiviral immune response by suppressing multiple inflammatory pathways.[10] So far, there have been no publications reporting the AT expression of NLRs in humans after weight loss. The aim of this study was to analyze the expression of subcutaneous adipose tissue (SAT) genes being 3 members of the NLR family, *NLRP12*, *NLRC3*, and *NLRX1*, before and after dietary intervention (DI) in relation to insulin sensitivity in individuals with obesity.

Patients and methods **Study group** A detailed study protocol was described in previous publications.[11,12] The study involved 28 participants with marked overweight or obesity (O/O) (body mass index [BMI] >28 kg/m^2; 16 women and 12 men; mean [SD] age, 34.7 [7.4] years). As a control group, we enlisted 11 participants with normal-weight (NW) (BMI <25 kg/m^2; 4 women and 7 men; mean [SD] age, 22.5 [1.4] years). All recruited individuals were nonsmokers, free of CVD,

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dyslipidemia, glucose intolerance / diabetes, hormonal dysfunction, hypertension, liver or renal failure, morbid obesity, neoplasms, or other serious conditions. The study participants did not use any blood pressure, glucose, hormone, or lipid-lowering medications. Patients were ineligible if they displayed clinical and / or laboratory indicators of inflammation or had been using anti-inflammatory medications within the past 3 months. The participants received clinical examination and relevant laboratory tests. Their body weight remained stable for at least 3 months prior to the study. In order to confirm that all participants had normal glucose tolerance, an oral glucose tolerance test was administered. Prior to the procedures, the participants underwent a 12-hour overnight fast. The Ethics Committee of the Medical University of Białystok approved the study protocol (R-I-002/28/2011), and all study participants provided their written informed consent.

Study protocol The O/O participants embarked on a 12-week DI program, which involved an individually tailored low-calorie diet (20 kcal/kg of ideal body weight, which was evaluated according to the Broca formula). The diet consisted of the following energy sources: 15%–20% protein, 25% fat, and 55%–60% carbohydrates. For each 2-week period, the participants were provided with instructions for a low-calorie diet along with a detailed daily menu. Qualified dieticians verified adherence to the diet and changes in body weight every 2 weeks. All analyses mentioned below were conducted both before and after the DI. The NW controls were assessed solely at baseline. The initial group consisted of 38 O/O and 20 NW individuals, but due to insufficient amount of available tissue, the expression of *NLRP12*, *NLRC3*, and *NLRX1* was only measured in 28 O/O and 11 NW individuals.

Insulin sensitivity measurement The measurement of insulin sensitivity was performed using the 2-hour hyperinsulinemic-euglycemic clamp. The M value, representing the rate of whole-body glucose uptake, was evaluated as the mean glucose infusion rate during the final 40 minutes of the clamp, adjusted for the glucose space and subsequently divided by fat-free mass (ffm).

Subcutaneous adipose tissue biopsy Using a biopsy needle under local anesthesia, SAT biopsy was acquired from the umbilical region, as described before.[11] The tissue was placed in a stabilization reagent (Allprotect Tissue Reagent, Qiagen, Hilden, Germany) and preserved at –80 °C until analysis.

Biochemical analyses Immediately after collection, the plasma glucose level was assessed with a glucose analyzer (YSI 2300 STAT PLUS, Yellow Springs Instruments, Yellow Springs, Ohio, United States). Serum lipids were measured via colorimetric assays using an autoanalyzer (Cobas C111, Roche Diagnostics, Mannheim, Germany). Serum concentrations of high-sensitive C-reactive protein (hs-CRP) were determined using particle-enhanced immunonephelometry (Dade Behring, Marburg, Germany).[11]

Analysis of mRNA expression of nuclear factor κB inhibitors RNA extraction and subsequent analysis of gene expression were performed following a previously described protocol.[11] The Turbo DNA-free Kit (Ambion, Austin, Texas, United States) was used to isolate RNA. The quantity and quality of RNA were verified using an Agilent Technologies 2100 Bioanalyzer and RNA 6000 Nano LabChip kit (Agilent, Santa Clara, California, United States). RNA purity was evaluated using a NanoDrop spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific Inc., Waltham, Massachusetts, United States). Reverse transcription was conducted using the QuantiTect Reverse Transcription kit (Qiagen, Hilden, Germany). Gene expression of the NFκB inhibitors was analyzed with quantitative polymerase chain reaction using a QuantStudio 6 system (Thermo Fisher Scientific Inc.) along with specific assays (*NLRC3*, Hs01054716_m1; *NLRP12*, Hs00536435_m1; *NLRX1*, Hs00226360_m1) following the manufacturer's instructions (TaqMan Gene Expression Assays, Thermo Fisher Scientific). The samples were standardized to the *PGK1* gene (Hs99999906_m1), as it exhibited the highest level of stability among the housekeeping genes examined. The relative mRNA expression was presented as fold-change using the ΔΔCt formula.

Statistical analysis Statistical analysis was carried out using STATISTICA 13.3 software (StatSoft, Kraków, Poland). Continuous variables are presented as arithmetic mean (SD) or geometric mean (geometric SD factor) for variables without normal distribution (gene expression, triglycerides, hs-CRP). The variables without normal distribution were log-transformed prior to the analysis and achieved normal distribution. The data without log transformation are shown in the Results section. Differences between the 2 groups were evaluated using the unpaired *t* test. Analysis of covariance was used to adjust for age and sex. The paired *t* test was used for estimation of differences in selected parameters before and after weight loss. Relations between the variables were examined with the Pearson product-moment correlation analysis. Multiple regression analysis was performed for adjustment of the observed relationships for BMI, age, and sex. For all observed correlations and differences, the Benjamini–Hochberg correction for multiple comparisons was applied. The level of significance was assumed at *P* value below 0.05.

Results The anthropometric and metabolic variables in NW and O/O individuals prior to the weight loss program are shown in

TABLE 1 Multiple regression analysis results with Δ insulin sensitivity and postintervention insulin sensitivity as dependent variables

Variable	Δ insulin sensitivity		Variable	Postintervention insulin sensitivity	
	Standardized β	*P* value		Standardized β	*P* value
Age	0.2	0.46	Age	–0.05	0.82
Sex (M1/F2)	–0.11	0.69	Sex (M1/F2)	0.24	0.31
Δ BMI	0.22	0.3	Postintervention BMI	–0.37	0.03
Δ *NLRP12*	0.57	0.01	Postintervention log*NLRP12*	0.42	0.01

Abbreviations: BMI, body mass index; F, female; M, male

Supplementary material, *Table S1*. By definition, O/O and NW participants showed disparities in anthropometric markers. The O/O patients displayed also lower insulin sensitivity, higher serum total cholesterol, higher concentrations of serum hs-CRP, triglycerides, lower high-density lipoprotein cholesterol and higher low-density lipoprotein cholesterol, and fasting glucose than the NW controls (Supplementary material, *Table S1*).

Prior to the DI, the O/O individuals exhibited lower AT expression of *NLRP12* than the NW individuals (mean [SD], 0.86 [1.78] vs 1.75 [1.62]; $P < 0.001$). The difference remained significant after adjustment for age and sex ($P = 0.03$). No significant differences were observed in the expression of *NLRC3* (mean [SD], 0.82 [1.97] vs 0.79 [2.19]) and *NLRX1* (mean [SD], 0.93 [1.49] vs 0.8 [1.35]) between the groups.

mRNA expression of nuclear factor κB inhibitors before and after the dietary intervention The DI resulted in a reduction in body weight (mean [SD], 101.13 [15.68] kg before the DI and 89.4 [14.03] kg after the DI; Δ = 11.73 kg; $P < 0.001$), improved insulin sensitivity (mean [SD], 6.09 [2.76] mg/kg_{ffm} × min before the DI and 8.15 [3.5] mg/kg_{ffm} × min after the DI; Δ = 2.06 mg/kg_{ffm} × min; $P = 0.002$), and reduced concentration of hs-CRP (mean [SD], 1.07 [2.55] mg/l before the DI and 0.7 [2.06] mg/l after the DI; Δ = 0.37 mg/l; $P < 0.001$).

In the post-DI group, we detected increased expression of *NLRP12* (mean [SD], 0.86 [1.78] vs 1.63 [1.69]; Δ = 0.77; $P < 0.001$), as compared with the O/O group before the DI. SAT *NLRP12* expression also increased in men and women analyzed separately (mean [SD], 0.89 [1.69] vs 1.51 [1.74]; $P = 0.04$ for men and 0.83 [1.87] vs 1.73 [1.67]; $P < 0.001$ for women). The expression of *NLRC3* (mean [SD], 0.82 [1.97] vs 0.9 [1.81]; $P = 0.92$) and *NLRX1* (mean [SD], 0.93 [1.49] vs 0.9 [1.29]; $P = 0.16$) did not change after the DI.

Correlations of *NLR12* mRNA expression with metabolic and anthropometric markers in normal weight and overweight/obese groups Before the DI program, there was no correlation between NFκB inhibitors and metabolic and anthropometric parameters with hs-CRP. Following the weight loss, the change in SAT *NLRP12* expression was negatively related to the changes in body mass and BMI ($r = –0.49$; $P = 0.009$ and $r = –0.49$; $P = 0.007$, respectively). The change in the SAT *NLRP12* expression was also related to a concurrent change in insulin sensitivity ($r = 0.48$; $P = 0.009$). After the DI, the SAT expression of log*NLRP12* was related to values of insulin sensitivity ($r = 0.48$; $P = 0.009$). The multiple regression analysis showed a correlation between the postintervention expression of log*NLRP12* and postintervention insulin sensitivity after DI that was independent from postintervention BMI, age, and sex (TABLE 1). The correlation between the changes in the *NLRP12* expression and the changes in insulin sensitivity after the DI was independent from the changes in BMI, age, and sex (TABLE 1).

Discussion Our study showed a decrease in SAT expression of *NLRP12* in O/O individuals, which was partially reversed by the DI. The expression of *NLRP12* correlated with insulin sensitivity after the weight loss program. We did not observe significant changes in the expression of other NFκB inhibitors (*NLRC3*, *NLRX1*). To the best of our knowledge, this is the first study to report AT expression of NLR receptors involved in regulating inflammation in humans after weight loss.

There was a significant increase in SAT expression of *NLRP12* after the DI. The data presented here suggest that the decrease in *NLRP12* expression can be partly reversed by diet-induced weight loss. The increase in *NLRP12* expression suggests that weight loss has a positive effect on this particular gene. This could imply that *NLPR12* plays a role in metabolic processes affected by weight. In 2018, Truax et al[13] found that obesity is associated with decreased expression of *Nlrp12* in murine AT. *Nlrp12* was shown to mitigate the impact of high-fat diet on inflammation and obesity through its influence on gut microbiota. Mouse mutants deprived of *Nlrp12* exhibited a greater weight gain and higher rate of AT inflammation.

The source of *NLRP12* in AT may be immune cells, as *NLRP12* is expressed in dendritic cells and macrophages, which regulate local inflammation. In the conditions such as obesity, adipocytes can undergo a transition to a proinflammatory state. This change also contributes to the proinflammatory state in AT.

In our experiment, we also observed a correlation between SAT *NLRP12* expression and insulin sensitivity after weight loss. A lack of correlation between the expression of *NLRP12* and

concentrations of hs-CRP, a well-established biomarker of inflammation, may suggest that the mechanism in which *NLRP12* affects insulin sensitivity operates independently of systemic inflammation. In previous research, our team assessed the effect of weight loss on insulin sensitivity and the SAT expression of genes associated with inflammation in patients with obesity. We found that the increase in insulin sensitivity resulting from weight loss was not accompanied by reduction in SAT inflammation.[9] The increased SAT expression of *NLRP12* may be an early change associated with local anti-inflammatory reaction.

We did not find any effect of weight loss on the expression of *NLRC3*. Additionally, there was no correlation between the expression of *NLRC3* and BMI or body mass. In multiple studies, short nuclear polymorphisms in the *NLRC3* gene have been associated with an increased risk of obesity and higher BMI.[15-18] Although the expression of *NLRC3* was not found to be connected with changes in insulin sensitivity, *NLRC3* is known to affect mTOR signaling, which can disrupt cellular metabolism, potentially leading to development of insulin resistance and obesity-related diseases.[18]

Similarly to *NLRC3*, the expression of *NLRX1* was unaffected by the weight loss program. It was also unrelated to BMI, body mass, or insulin resistance. In other publications, mice lacking the *Nlrx1* gene were partially protected from hyperglycemia induced by a high-fat diet, suggesting that *Nlrx1* might be involved in obesity-related conditions.[19]

The lack of change in the expression of *NLRX1* and *NLRC3* suggests that their functions may not be directly involved in the pathways affected by weight loss and insulin sensitivity. It is important to consider limitations of the experimental model, specific functions of the proteins encoded by these genes, and diverse responses of different proteins to weight loss when interpreting our results. Future research should aim to further clarify the role of *NLRX1* and *NLRC3* in insulin sensitivity, potentially exploring different models and experimental conditions to gain a better understanding of their functions in the context of metabolic health and weight loss.

The limitations of our study include inability to measure SAT protein expression. There was also a disparity in the age of NW and O/O individuals. Age and sex may potentially confound the results. However, it is noteworthy that all the observed differences between the groups remained significant after adjusting for age and sex. Another limitation of this study is the inability to establish causality.

In conclusion, our data suggest that weight loss leads to a change in SAT *NLRP12* expression, which is associated with an increase in insulin sensitivity. The mechanism behind this effect could be associated with the anti-inflammatory activity of *NLRP12*. *NLRP12* could be a potential target for treatment of obesity and associated metabolic disorders.

SUPPLEMENTARY MATERIAL

Supplementary material is available at www.mp.pl/paim.

ARTICLE INFORMATION

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