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The association of TNFr2 and TNF α with insulin resistance and the influence of adipose tissue biomarkers in humans

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Abstract

TNF α is a pro-inflammatory adipokine hypothesized to link obesity with insulin resistance. Functional studies suggest that TNF α act through pathways involving adipokines and fatty acids to induce insulin resistance. We tested the hypothesis that the association of measures of TNF α activity with insulin resistance is independent of obesity and adipose tissue biomarkers. We analyzed data from 2,131 participants (without diabetes) of the Framingham Offspring Study exam 7. The outcome of interest was insulin resistance, measured using the homeostasis model (HOMA-IR). TNF α activity was measured by plasma TNFr2 or TNF α ; possible cofounders included adipose tissue biomarkers (plasma adiponectin, resistin, and triglycerides). We used multivariable age- and sex-adjusted linear regression analyses to adjust for waist circumference, biomarkers individually, simultaneously, and in biomarker-stratified (above and below median) models. We found that TNFr2 was positively associated with HOMA-IR ($r = 0.21$, $P < .0001$). In age- and sex-adjusted model, each increase of one SD of TNFr2 (SD=746 pg/mL), the log (HOMA-IR) value was increased by 0.11 units ($p < .0001$). Adjustment for waist circumference reduced the TNFr2 beta coefficient (by about 45%) but the association between TNFr2 and HOMA-IR remained significant ($p < .0001$). TNFr2 was still associated to HOMA-IR after adding adiponectin, resistin, and triglycerides (individually, and simultaneously). We found similar associations with plasma levels of TNF α . We conclude that in a

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representative community sample, measures of TNF α activity are associated with insulin resistance, even after accounting for central adiposity and other adipose tissue biomarkers.

Keywords

Tumor necrosis factor alpha; tumor necrosis factor alpha receptor; adiponectin; resistin; triglycerides; biomarkers; insulin resistance

1. Introduction

Adipose tissue is recognized to be an energy storage tissue as well as an endocrine tissue that secretes proteins known as adipokines. It has been proposed that excess fat accumulation alters the pattern of adipokines secretion causing a low-grade pro-inflammatory state that induces insulin resistance and endothelial dysfunction, leading to diabetes and cardiovascular disease (1). TNF α is produced primarily by adipose-infiltrating macrophages (2) and was one of the first proteins to be identified as part of the adipose tissue inflammatory pathways (3). In circulation, TNF α is bound to a number of circulating proteins, including the soluble form of TNF α receptor 2 (TNFr2) which might act as a buffer system (or slow-release reservoir) for TNF α (4). Increased circulating levels of both TNF α (3,5) and TNFr2 (6,7) have been associated with markers of insulin resistance.

Based on *in vitro* data and animal studies, several pathways have been proposed to explain how TNF α could induce insulin resistance (2,5). TNF α decreases adipocyte production of adiponectin (8), an adipokine with anti-inflammatory and insulin-sensitizing properties (9). TNF α also induces the production of pro-inflammatory proteins including resistin, an adipokine secreted mainly by macrophages in human adipose tissue (10,11). Another potential mechanism linking TNF α to insulin resistance is related to TNF α action on lipid regulation and adipocyte lipolysis. In mouse models fed a high-fat, high calorie diet, TNF α knock-out protects against the development of hyperinsulinemia (12), whereas rats given a TNF α infusion (causing insulin resistance) showed an increase in both circulating non-esterified fatty acids (NEFA) and triglycerides (13). In fact, these diverse adipokine inter-relations are likely to be all involved in the pathophysiology of insulin resistance.

Data linking TNF α or TNFr2, other adipose tissue-derived pathways, and insulin resistance have come primarily from *in vitro*, animal model, and small physiological studies in humans, but there is less evidence that these relations operate at the population level. Using data from the seventh examination of the community-based Framingham Offspring Study, we have shown previously that TNF α is associated with insulin resistance, even after adjustment for adiposity (14). To address the question of adipokine inter-relations, we conducted analyses to test the hypothesis that the relation between measures of TNF α activity (plasma TNFr2 or TNF α) and insulin resistance is influenced by biomarkers (adiponectin, resistin, triglycerides) reflecting different adipose tissue pathways.

2. Methods

2.1. Study participants

Started in 1971, the Framingham Offspring Study is a community-based, prospective, observational study following the children of the original Framingham Heart Study cohort, and their spouses, to investigate cardiovascular disease and its risk factors (15). At baseline 5,124 participants, mainly white, of European ancestry, were recruited. Participants were invited to return for follow up measurements on a periodic basis. We used data from the seventh examination cycle, conducted between 1998 and 2001 (n=3,539 attendees). Evaluation

included fasting blood samples, a standardized medical examination, and a medical history. For this analysis, we excluded participants with prevalent diabetes (n=389). We present here analysis of the participants (total n=2061) with both TNFr2 and TNF α levels measured, after excluding individuals with missing values for adipokines levels (n=483) or metabolic risk factors (n=311). The study protocol was approved by the Institutional Review Boards of the Boston University Medical Center and of the Massachusetts General Hospital; all participants provided written informed consent.

2.2. Exposure and outcome definitions

The primary outcome of interest was insulin resistance, measured using the homeostasis model (HOMA-IR, calculated by [fasting glucose $\times$ fasting insulin]/22.5) (16), which was considered as a continuous variable. Activity of the TNF α pathway was represented by measurements of circulating plasma TNFr2 or TNF α . Potential confounders included adipose tissue biomarkers (adiponectin, resistin, and triglycerides), which we chose because literature suggested that they reflect the potential pathways through which TNF α could induce insulin resistance. We used levels of triglycerides as a proxy for NEFA flux. In humans, elevated plasma triglycerides reflect increased NEFA flux: in physiological studies, rising plasma NEFA increased circulating triglycerides (17); circulating NEFA and triglycerides are modestly correlated in population studies (18), and hypertriglyceridemia is a well known characteristic of insulin-resistant states (19).

Laboratory methods for glucose, insulin, and lipid assays have been published previously (20). Fasting plasma glucose was measured immediately with a hexokinase reagent kit (A-gent glucose test, Abbott, South Pasadena, CA), and other plasma samples were frozen at -80°C until assay. Fasting plasma insulin was measured with a human specific insulin assay having essentially no cross-reactivity to proinsulin or insulin split-products (Linco Inc., St Louis, MO). Triglycerides levels were determined by commercial assay (Abbott Spectrum CCX, South Pasadena, CA). Total adiponectin, resistin, TNFr2 and TNF α were measured by ELISA (R&D Systems, Minneapolis, MN). We used a high sensitivity TNF α assay in order to capture variation in TNF α in the very low levels that we expected find in a community-based sample. In our lab, intra-assay coefficients of variation (CV) were $<3\%$ for glucose, 6.1% for insulin, 1.2% for triglycerides, 5.8% for adiponectin, 9.0% for resistin, 2.2% for TNFr2 and 6.6% for TNF α . Based on the assay kit we used (R&D Systems), inter-assay values and sensitivities were 5.8–6.9% and 0.246 ng/ml for adiponectin; 7.8–9.2% and 0.026 ng/ml for resistin; 3.5–5.1% and 0.6 pg/ml for TNFr2; and 6.3–8.3% and 0.391 pg/ml for TNF α .

Other covariates included standardized physical exam measures. We measured height, weight, and waist circumference (at the umbilicus) with the subject standing. We calculated body mass index (BMI) as weight in kilograms divided by the square of height in meters (kg/m^2).

2.3. Statistical analysis

We used means and standard deviations to describe continuous characteristics and percentages for categorical characteristics. Variables not normally distributed were log-transformed. We used Pearson correlations to assess associations between TNFr2 or TNF α and various clinical and biochemical variables. For linear regression we modeled HOMA-IR as a continuous variable and the adipokines as per change of one standard deviation to be able to compare their relative effects on a unit change in HOMA-IR. We performed all analyses using circulating TNFr2 or TNF α as markers of TNF α activity, but we present results from TNFr2 in the main manuscript because of our results with Pearson correlations (showing stronger associations with metabolic traits) concordant with previous literature (6,7). We present all the results using TNF α in online supplementary tables. We first tested the age- and sex-adjusted association of TNFr2 with HOMA-IR. Knowing that both TNFr2 and insulin resistance are related to central

adiposity, we added waist circumference to the model to assess the impact of adjusting for adiposity on the TNFr2-HOMA-IR association. We then tested the effect of adding adipose tissue biomarkers (adiponectin, resistin, and triglycerides) individually and together to the models adjusted for age, sex and waist circumference. We assessed whether adding adipose tissue biomarker variables to the models altered the size, direction or statistical significance of the TNFr2 beta coefficient association with HOMA-IR.

We then stratified the analysis to examine whether the adipose tissue-derived biomarkers modified the effect of TNFr2 on insulin resistance by testing the association in individuals with low or high levels of adipose biomarkers. We divided the TNFr2 distribution by tertiles and examined the level of HOMA-IR in each tertile by two strata of adipose tissue biomarker defined as below or above the median level. We examined the trends within each stratum, and tested statistical significance of TNFr2-biomarker interactions using first-order interaction terms in multivariable linear regression models. We adjusted all the stratified analysis for age, sex and waist circumference. We considered P values less than 0.05 to indicate statistical significance. We performed all analyses using SAS software (version 8.1, SAS Institute, Cary, NC).

3. Results

3.1. Participant characteristics

The characteristics of the Framingham Offspring Study participants are presented in Table 1. At exam 7, participants were on average 61 years old, mean BMI was in the overweight range, and about half were women. Pearson correlations of (log-transformed) TNFr2 and TNF α with several clinical and biochemical factors are shown in Table 2. Circulating levels of TNFr2 and TNF α were modestly correlated with each other ($r = 0.36$, $P < .0001$). In general, the correlations were stronger with TNFr2 than with TNF α levels. The characteristics that showed the strongest associations with TNFr2 were age ($r = 0.39$; $P < .0001$) and resistin levels ($r = 0.31$; $P < .0001$).

3.2. Multivariate models: TNF α measures association with insulin resistance

In a series of linear regression models we estimated the relation of adiposity and biomarkers with the association of TNFr2 with HOMA-IR. As shown in Table 3, age- and sex-adjusted levels of TNFr2 were associated with HOMA-IR. Adjusting for waist circumference, the beta coefficient for TNFr2 decreased substantially (going from 0.11 to 0.06; a 45% decrease) but TNFr2 remained significantly associated with HOMA-IR. We then tested the influence of adding adiponectin, resistin, or triglycerides (one at a time) on the TNFr2-HOMA-IR association. The age-sex-and-waist-adjusted beta coefficient for TNFr2 was barely influenced by adding biomarkers individually. When modeling the biomarkers simultaneously, the age-sex- and waist-adjusted TNFr2 beta coefficient was still significantly associated with HOMA-IR. Adiponectin and triglycerides levels were also significantly associated with HOMA-IR in the fully-adjusted models, but not resistin levels.

We conducted the same analyses using TNF α instead of TNFr2 (see Online Supplement Table 1). In all the models, TNF α was significantly associated with HOMA-IR, and the beta coefficient was influenced in a similar fashion with the successive adjustments. The association remained significant ($p = .04$) in the full model that included waist circumference and all three adipose tissue biomarkers.

3.3. Effect of biomarker stratification on the TNFr2-HOMA-IR association

Low adiponectin, high resistin, and high triglycerides levels have been associated with a higher-risk metabolic profile. We tested if the TNFr2-HOMA-IR association was modified in individuals with adverse levels of these adipose tissue biomarkers. Figure 1 shows HOMA-IR

values by TNFr2 tertiles, stratified by below or above median of the adipose tissue biomarkers (all analyses adjusted for age, sex, and waist circumference). The associations between TNFr2 and HOMA-IR remained significant in all the strata of biomarkers. No biomarker-TNFr2 interactions were statistically significant (all $p > .05$), but the individuals with resistin levels above the median tended to show a stronger association between TNFr2 and HOMA-IR (interaction p -value=.08).

4. Discussion

The association of insulin resistance with TNF α has been demonstrated *in vitro* and in animal studies, but has been less consistently shown in human studies (21–24). We have shown previously that TNF α levels were associated with insulin resistance, adjusting for adiposity and metabolic syndrome status (14). Here we have shown that HOMA-IR and other metabolic traits, including adipose tissue biomarkers, were correlated with circulating levels of TNF α , but showed stronger correlations with circulating TNFr2 levels in general, as a marker of TNF α activity. TNFr2 is believed to stabilize circulating TNF α and to act as a “slow release reservoir” of bio-active TNF α (4). In accordance with our data, TNFr2 has been shown to have similar or stronger correlations with insulin resistance compared to circulating TNF α levels (7). We extend understanding of the association between TNF α pathways and insulin resistance by taking into account circulating levels of adiponectin, resistin, and triglycerides, representing mediating pathways by which TNF α could act based on functional studies (2,5). Our report lends support to the concept that TNF α is involved in the pro-inflammatory process linking adiposity to development of diabetes not only in animal models but also in community-dwelling adults.

4.1. Extending understanding of functional studies of adipose tissue

Numerous functional studies of adipose tissue have outlined the complex interactions of adipokines produced by adipocytes and macrophages. Adipocytes are the major triglyceride storage site and have the exclusive ability to secrete adiponectin (25). Resistin and TNF α can be secreted by both adipocytes and macrophages, but in humans these adipokines seem to be mainly derived from adipose tissue infiltrating macrophages (2,26).

It has been shown that adiponectin decreases TNF α production in macrophages (27), and that TNF α decreases adiponectin expression (8,28) and secretion (29,30) in adipocytes, leading to a vicious pro-inflammatory cycle. In our data, adiponectin was weakly associated with TNF α and TNFr2 ($r = -0.06$ and $r = -0.05$ respectively), had minimal impact when added to the age-, sex-, and waist-adjusted models, and was still significantly associated with insulin resistance in fully-adjusted models. This suggests that adiponectin and TNF α provide complementary information and both influence pathways leading to insulin resistance.

Resistin was the biomarker with the strongest association with both TNFr2 and TNF α levels. We have shown in the past that resistin levels are associated with insulin resistance, even when accounting for adiposity (14), but this association was not significant in the present analysis when including TNFr2 levels and central adiposity in the models. This could be due to the fact that in human adipose tissue both resistin and TNF α are produced by infiltrating macrophages and so could be participating in closely linked pro-inflammatory pathways. *In vitro*, resistin stimulates production of TNF α by human adipocytes (31) and macrophages (32). TNF α in turn can stimulate resistin in blood mononuclear cells (10,11). A possible pro-inflammatory feed forward loop is supported by our data pointing to a stronger association between TNFr2 and HOMA-IR in individuals with high resistin.

In vitro, non-esterified fatty acids (NEFA) have been shown to decrease adiponectin secretion and to increase TNF α secretion in adipocytes (33). In macrophages, saturated fatty acids

increase TNF α expression (2). In addition, co-cultures of adipocytes and macrophages induced an increased production of NEFA, which could be decreased by blockage of TNF α action (2). In animal models, TNF α infusion increased circulating plasma NEFA after 1 day of infusion and increased both plasma NEFA and triglycerides after 4 days of TNF α infusion (13). Using triglycerides as a proxy for NEFA flux, we found that TNFr2 was associated positively with triglycerides and that both biomarkers were independently associated with HOMA-IR in multivariable models. Overall, we found evidence supporting insulin resistance-TNF α -adipokine associations in adults in the community, with directions of effects that were expected on the basis of known molecular and physiologic relationships.

4.2. Markers of TNF α activity and other adipokines in population-based studies

Population-based studies exploring the interrelations of diverse adipokines and inflammatory markers and their effect on insulin resistance are scarce, and few have considered a comprehensive set of biomarkers. Circulating TNF α levels have been inconsistently associated with insulin resistance in small cohorts, where some have found significant associations (21, 24) whereas others have not (22,23). We previously reported that elevated TNF α levels were associated with increased insulin resistance (14); here, we took advantage of our large sample size to further characterize the influence of key adipose tissue biomarkers on this association. We found modest significant correlations markers of TNF α activity with adiponectin, resistin, and triglycerides of similar magnitude to those reported in another general population-based study of Chinese men (34). Few population studies have simultaneously assessed more than one biomarker in relation to insulin resistance. In a Swedish male cohort, TNF α and adiponectin levels were both associated with glucose infusion rate assessed by euglycemic hyperinsulinemic clamps (21). Population studies have shown a significant positive correlation between resistin and TNF α (35,36), but did not model both in relation to insulin resistance.

4.3. Strength and limitations

Strengths of this study include a large sample size from a community-based cohort, clinical measurements taken under standardized protocols, and a comprehensive set of biomarkers measured using assays with good precision. We present results using TNFr2, which is known to represent a good reflection of TNF α activity (4,6,7). We confirmed our finding by repeating the analysis using TNF α , measured with a high-sensitivity assay that detected low concentrations of the biomarker in the ranges typical of community-based sample. A limitation of our study is that formal power to detect statistical interactions might not have been sufficient in some of the stratified analyses. Another limitation includes the use of surrogate markers for insulin resistance (HOMA-IR) and for NEFA (triglycerides). We recognize that triglycerides can be influenced by many clinical situations, are not a direct measure of circulating fatty acids in individuals and that triglycerides cannot reliably replace direct measurement of circulating fatty acids. As circulating fatty acids are not available in the Framingham Offspring Study, we used triglycerides as a surrogate of fatty acid flux. Like HOMA-IR is not a precise measure of insulin resistance in individuals but does discriminate insulin resistance from insulin sensitive groups at the population level, we used triglycerides to define groups with relatively low versus relatively high free fatty acid flux at the population level. If anything, this might have reduced our ability to observe associations and our results may be underestimations of the true associations. We measured only total adiponectin and not the high molecular weight fraction, which has been proposed to have a stronger correlation with insulin resistance and metabolic syndrome compared with total adiponectin (37,38). Some might raise the fact that we used a single measurement of each biomarker; but one point adipokine measurement has been shown to be reliable compared to more than one assessment over many seasons (34). Further limitations include the cross-sectional design of our analyses which does not allow us to make conclusions regarding causal direction. Finally, the Framingham cohort is largely white and

middle-aged or older, so findings may have limited generalizability to other ethnic and age groups.

In conclusion, we have shown in a large community-based sample that markers of TNF α activity are associated with insulin resistance, even after taking into account central adiposity status and other adipose tissue biomarkers. Our observations in this representative population add to the functional studies suggesting that anti-inflammatory, pro-inflammatory, and lipolytic pathways are implicated in the association of TNF α with insulin resistance. Prospective follow up and physiological studies are needed to help us untangle the causal relations and to understand the sequence of adipokines interactions leading to insulin resistance and diabetes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

TNF α	tumor necrosis factor alpha
TNFr2	tumor necrosis factor alpha receptor 2
NEFA	non-esterified fatty acids
HOMA-IR	homeostasis model assessment of insulin resistance
ELISA	enzyme-linked immunosorbent assay
BMI	body mass index

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Figure 1.

Top panel: Insulin resistance measured by HOMA-IR in each TNFr2 tertile, further stratified by adiponectin level (below or above median). All data is adjusted for age, sex, and waist circumference. P-values for trend of association with HOMA-IR across TNFr2 tertiles are shown in the figure; $P=.51$ for interaction (TNFr2 by adiponectin predicting HOMA-IR).

Middle panel: Insulin resistance measured by HOMA-IR in each TNFr2 tertile, further stratified by resistin level (below or above median). P-values for trend of association with HOMA-IR across TNFr2 tertiles are shown in the figure; $P=.08$ for interaction (TNFr2 by resistin predicting HOMA-IR). **Bottom panel:** Insulin resistance measured by HOMA-IR in each TNFr2 tertile, further stratified by triglyceride (TG) level (below or above median). P-

values for trend of association with HOMA-IR across TNFr2 tertiles are shown in the figure; $P=.60$ for interaction (TNFr2 by TG predicting HOMA-IR).

Table 1

Characteristics of 2,131 participants free of diabetes at Framingham Offspring Study examination 7

	mean (SD) or %
N	2061
Women (%)	51
Age (years)	61 (10)
Body mass index (kg/m ²)	27.8 (5.1)
Waist circumference (inches)	97.3 (13.3)
HOMA-IR	3.86 (2.46)
TNF α (pg/mL)	1.45 (1.26)
TNFr2 (pg/mL)	2120 (746)
Adiponectin (μ g/mL)	10.48 (6.56)
Resistin (ng/mL)	14.09 (7.14)
Triglycerides (mg/dL)	132.9 (82.0)

HOMA-IR: homeostasis model of insulin resistance

Table 2

Pearson correlation coefficients between TNF α or TNFr2 and clinical or biochemical factors in 2,131 participants free of diabetes at Framingham Offspring Study examination 7

Clinical and biochemical factors	Correlation with TNF-r2	<i>P</i>	Correlation with TNF α	<i>P</i>
n	2,061		2,131	
Age	0.39	<.0001	0.19	<.0001
BMI	0.19	<.0001	0.07	.001
Waist circumference	0.22	<.0001	0.09	<.0001
Triglycerides	0.12	<.0001	0.09	<.0001
Adiponectin	−0.05	.01	−0.06	.009
Resistin	0.31	<.0001	0.20	<.0001
HOMA-IR	0.21	<.0001	0.09	<.0001
TNF α	0.36	<.0001	-	-

BMI: body mass index, HOMA-IR: homeostasis model of insulin resistance

Table 3

Association TNFr2 with HOMA-IR in the Framingham Offspring Study, adjusted for age and sex, further adjusted for waist circumference and adipose tissue biomarkers.

	TNFr2	Adiponectin	Resistin	Triglycerides
Age- and sex-adjusted				
Beta (SE) ^a	0.11 (0.01)			
p-value	<.0001			
Age- sex- and waist circumference-adjusted				
Beta (SE)	0.06 (0.01)			
p-value	<.0001			
Age, sex and waist circumference adjusted; adding adipose tissue biomarkers individually				
Beta (SE)	0.06 (0.01)	−0.12 (0.01)		
p-value	<.0001	<.0001		
Beta (SE)	0.05 (0.01)		0.02 (0.01)	
p-value	<.0001		.12	
Beta (SE)	0.05 (0.01)			0.11 (0.01)
p-value	<.0001			<.0001
Age-, sex- and waist-circumference adjusted, adding all three adipose tissue biomarkers simultaneously ^b				
Beta (SE)	0.05 (0.01)	−0.10 (0.01)	0.01 (0.01)	0.09 (0.01)
p-value	<.0001	<.0001	.28	<.0001

HOMA-IR: homeostasis model of insulin resistance

^a beta coefficient in linear regression models predicting HOMA-IR (log-transformed) reflect a standard deviation (SD) change in each biomarker. By example in the age- and sex-adjusted model, for each increase of one SD of TNFr2 (SD=746pg/mL, non-transformed), the log (HOMA-IR) value would be increased by 0.11 units.

^b estimated variance of the full model: r²=0.36