Circulating Cytokines Predict the Development of Insulin Resistance in a Prospective Finnish Population Cohort

Kristiina Santalahti, Mikael Maksimow, Antti Airola, Tapio Pahikkala, Nina Hutri-Kähönen, Sirpa Jalkanen, Olli T. Raitakari, and Marko Salmi

MediCity Research Laboratory and Department of Medical Microbiology and Immunology (K.S., M.M., S.J., M.S.), University of Turku, Turku, Finland; Department of Information Technology (A.A., T.P.), University of Turku, Turku, Finland; Department of Pediatrics (N.H.-K.), University of Tampere and Tampere University Hospital, Tampere, Finland; Research Centre of Applied and Preventive Cardiovascular Medicine (O.T.R.), University of Turku and Department of Clinical Physiology and Nuclear Medicine, Turku University Hospital, Turku, Finland

Context: Metabolic inflammation contributes to the development of insulin resistance (IR), but the roles of different inflammatory and other cytokines in this process remain unclear.

Objective: We aimed at analyzing the value of different cytokines in predicting future IR.

Design, Setting, and Participants: We measured the serum concentrations of 48 cytokines from a nationwide cohort of 2200 Finns (the Cardiovascular Risk in Young Finns Study), and analyzed their role as independent risk factors for predicting the development of IR 4 years later.

Main Outcome Measures: We used cross-sectional regression analysis adjusted for known IR risk factors (high age, body mass index, systolic blood pressure, triglycerides, smoking, physical inactivity, and low high-density lipoprotein cholesterol), C-reactive protein and 37 cytokines to find the determinants of continuous baseline IR (defined by homeostatic model assessment). A logistic regression model adjusted for the known risk factors, baseline IR, and 37 cytokines was used to predict the future IR.

Results: Several cytokines, often in a sex-dependent manner, remained as independent determinants of current IR. In men, none of the cytokines was an independent predictive risk marker of future IR. In women, in contrast, IL-17 (odds ratio, 1.42 for 1-SD change in ln-transformed IL-17) and IL-18 (odds ratio, 1.37) were independently associated with the future IR. IL-17 levels also independently predicted the development of incident future IR (odds ratio, 1.48).

Conclusions: The systemic levels of the T helper 1 cell cytokine IL-18 and the T helper 17 cell cytokine IL-17 thus may have value in predicting future insulin sensitivity in women independently of classical IR risk factors. **(*J Clin Endocrinol Metab* 101: 3361–3369, 2016)**

Insulin resistance (IR) is a key element in the pathogenesis of metabolic syndrome and type 2 diabetes (1, 2). Development of IR has been linked to chronic, obesity-induced inflammation, which is reflected by elevated systemic cytokine levels. In mouse models, certain proinflammatory cytokines are causally involved in the development of IR. For instance, mice deficient in TNF-α, TNF receptor (3), or IL-1 receptor (4) all have improved insulin sensitivity. Mechanistically, TNF-α inhibits the phosphorylation of insulin receptor substrate 1 (5), whereas IL-1β reduces insulin receptor substrate 1 expression (6). Many proinflammatory cytokines can also indirectly in-

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Abbreviations: AUC, area under the curve; BMI, body mass index; BP, blood pressure; CCL, chemokine (C-C motif) ligand; CI, confidence interval; CRP, C-reactive protein; HDL, high-density lipoprotein; HGF, hepatocyte growth factor; HOMA, homeostatic model assessment index; IR, insulin resistance; OR, odds ratio; Th1, T helper 1 cell; Th17, T helper 17 cell.

duce IR by causing induction of inflammatory genes, which then alter glucose uptake and insulin sensitivity (7). However, the clinical relevance of cytokines to the pathogenesis of IR is still not clear, because intervention studies using anticytokine treatments against proinflammatory cytokines have given unexpected and complex results (8). For instance, multiple anti-TNF-α trials have failed to show effect on glycemic control (9–11), whereas neutralization of IL-1β improves glycaemia (12, 13).

In humans, IR has been correlated with TNF-α, IL-1β, IL-6, and chemokine (C-C motif) ligand (CCL) 2 levels in several studies (14, 15). However, most human studies have included rather low numbers of participants, confounding factors have not been rigorously addressed, and only a few cytokines at the time had been analyzed. Moreover, the ability of circulating cytokines to predict the development of IR has not been studied.

The aim of our study was to explore the role of inflammatory markers in explaining current and future IR. Using high-throughput techniques we simultaneously analyzed 48 cytokines from the blood samples of 2200 young Finns. Using this prospective cohort, we studied the correlations of cytokines with IR, body mass index (BMI), and C-reactive protein (CRP), and determined which cytokines could predict future IR.

Materials and Methods

Study design and subjects

The Cardiovascular Risk in Young Finns Study is a cohort study conducted in five Finnish university hospitals (Turku, Helsinki, Tampere, Oulu, Kuopio) since 1980 (16). In this study, we used the blood samples and data from the 27-year follow-up (2007) and 31-year follow-up (2011). A total of 2200 subjects participated in the 27-year follow-up. We excluded participants who were pregnant (n = 37) or had diagnosed type 1 or type 2 diabetes (n = 16 and 17, respectively), leaving 2130 for the current study population. A total of 2060 persons participated to the 31-year follow-up study. Of these, 1732 had also participated in 2007 study; of these, 1723 persons had glucose and insulin measurements from the year 2011. Only those participants who had all the variables available were included in the analysis in the question. Local ethical committees have approved the study protocol and all study participants provided written informed consent.

Laboratory measurements

The height (m) and weight (kg) were measured and BMI (kg/m^2) was determined. The systolic blood pressure (BP) (BP; mm Hg) was measured at a supine position using an automatic manometer (random zero). High density lipoprotein (HDL) cholesterol (mmol/liter), triglycerides (mmol/liter), fasting plasma glucose (mmol/liter), insulin (mU/liter), and high-sensitivity CRP (mg/liter) were measured, as described (17).

Clinical characteristics

The following characteristics were self-reported on a structured questionnaire: age, pregnancy, diagnosis of type 1 and type 2 diabetes, smoking (never/less than daily vs daily), and physical activity (combination of intensity, duration and quality of workout, total number and length of physical activity, values from 5 to 15 [the higher value indicates a more active person]). In this study, homeostatic model assessment (HOMA index; insulin-glucose product divided by 22.5, unit mU · mmol/liter^2) was used to define the IR (18). Future IR consisted of the subjects who had IR higher than the 90th percentile in continuous IR in 2011 (3.97 mU · mmol/liter^2 and 4.78 mU · mmol/liter^2 for women and men, respectively). Incident IR consisted of only those subjects who were not in the top 10% in baseline (2007) IR, but were in the top 10% in 2011 IR.

Measurement of cytokines, chemokines, and growth factors

After overnight fasting (≥12 hours), the sera were drawn and stored at –70 °C until the measurement of the cytokines. The concentrations of 48 cytokines, chemokines, and growth factors (names and abbreviations listed in the Supplemental Data (Supplemental Table 1) were measured from 2200 serum samples with Bio-Rad's premixed Bio-Plex Pro Human Cytokine 27-plex Assay (catalog no. M500KCAF0Y) and 21-plex Assay (catalog no. MF0005KMII) kits on Bio-Rad's Bio-Plex 200 System. All assays were made according to the manufacturer's instructions except that the amount of beads, detection antibodies, and streptavidin-phycoerythrin conjugate were used at half of their recommended concentration; this was determined to be sufficient in preliminary tests. With the exception of IL-12(p40) and IL-12p(70), and macrophage inflammatory protein-1α and macrophage inflammatory protein-1β, all analytes demonstrated less than 2% cross-reactivity (19). Eight-point standard curves were generated for each cytokine using recombinant proteins. The values falling outside the standard range were manually extrapolated according to the standard curve of the given plates. After this, all the values that deviated more than 10 SDs from the mean were given the next smallest or largest value. Two of the analytes (CCL5 and IFN-α2) gave values outside the standard range, and were thus not included in the analysis. The standard range and the intra-assay and interassay variations are shown in Supplemental Table 1.

Statistical analysis

All continuous variables are shown as medians (25th–75th percentile). Class variables are given as numbers of individuals in percentages. All analyses were done sex-wise.

Mann-Whitney *U* test was used to compare the distributions of continuous variables. Spearman correlation was used to study associations of continuous variables. In all the univariate analyses, the significance level was divided by the number of the performed tests to rigorously compensate for the multiple testing.

For the regression models, all the continuous variables were first ln-transformed and then standardized (mean = 0, SD = 1). Linear regression analysis was used to study the cross-sectional associations of the variables with IR. The variables were chosen by using a stepwise selection method in which variables are added to the model one by one based on their contribution to the model (the variable's F statistics must be significant at threshold

level of $P < .05$), and after each step all the variables in the model must produce a F statistic significant at the threshold level of $P < .05$. The model variables were chosen from all the variables classically associated to IR (age, BMI, triglycerides, HDL cholesterol, systolic BP, smoking, and physical activity), CRP, and all the cytokines. The results are expressed as parameter estimates for a 1-SD increase in continuous variables and one category change in categorical variables. The inter correlations of the cytokines was minimized in the model by using stepwise selection method and by checking the tolerance and variation inflation of the factors. The validity of the model was assessed by observing the regression residuals.

Logistic regression analysis was used to study the association of cytokines with the odds of outcome (persons in the top 10% of IR calculated from the 2011 HOMA index). In addition to the baseline variables included in the model (the same as discussed previously), the baseline (from year 2007) IR was included to the list of variables available for the stepwise selection method. The results are shown as odds ratios (OR) with 95% confidence intervals (CI) of standardized ln-transformed continuous variables. The stability of the model building and the results were studied with 5-fold cross-validation by randomly dividing the data into five equal parts. Each training fold (comprising four of five parts) was then used for model variable selection as well as for obtaining the parameter estimates.

To estimate the model performance in predicting future IR, 5-fold cross-validated area under curve (AUC) was calculated. Two models were compared, the standard model (model variables to choose: baseline IR, age, BMI, CRP, triglycerides, HDL cholesterol, systolic BP, smoking, and physical activity) and the new model (model variables to choose: those previously mentioned and 37 cytokines). The model building was done in each of the five training folds (each comprising 4/5 of the data). The intercepts and parameter estimates acquired from a training fold were then used in the test fold (the remaining 1/5 of the data, which was not used in the model building). The prediction results were then pooled and AUCs were calculated.

Statistical analyses were done using SAS version 9.4 (SAS Institute Inc.), the pROC package (version 1.7.3) for the R project for the Statistical Computing, and custom built software implemented in the Python language (version 2.7.6).

Results

Cytokine concentrations and other baseline characteristics of the baseline study population

A total of 2200 persons participated to the 27-year follow-up of the Cardiovascular Risk in Young Finns Study in year 2007. From these, the serum concentrations of 48 cytokines were measured. The success rate (the percentages of measurements, in which the cytokine concentration was within the range of the 8-point standard curve) of each cytokine measurement, is shown in Supplemental Table 1. Even though the values falling outside the standard range were manually extrapolated, only the cytokines which gave a valid concentration within the standard range for more than 90% of the samples (37 cytokines) were included in the subsequent analyses.

After the exclusions resulting from pregnancy (n = 37) or type 1 or 2 diabetes (n = 16 and n = 17, respectively), 2130 participants remained in the baseline study population and were eligible for the subsequent analysis. Most of the clinical and laboratory measurement values had different levels in women and men (Supplemental Table 2). In addition, half of the cytokines, including nearly half of the chemokines and growth factors, were found at different levels in men and women, and the interaction terms were significant in the subsequent models. Therefore, in this study all analyses were done separately for men and women.

Cytokines associate with IR and multiple other baseline characteristics

We first analyzed which of the cytokines correlate with baseline IR (Table 1). In univariate analyses, IL-18, CCL4, and hepatocyte growth factor (HGF) had strong direct correlations and CCL27 the strongest indirect correlation to IR in both sexes. The rest of the associations were only seen in either of the sexes. Especially, tumor necrosis factor-related apoptosis inducing ligand and macrophage migration inhibitory factor in men, and IL-2rα, IL-5, IL-10, IL-13, IL-7, and vascular endothelial growth factor in women, were associated to higher IR.

In addition to the IR, cytokines had also correlations to other continuous baseline characteristics. In women, all growth factors, except stem cell factor, had a direct univariate association to CRP (Supplemental Table 3). Eighty-one percent of all cytokines correlated with triglycerides; this was particularly evident with interleukins and growth factors. Nearly half of the cytokines had a correlation to BMI and more than one-third of cytokines to systolic BP. In men, in contrast, only a few correlations were evident, most of them to CRP (Supplemental Table 4). As expected, cytokines also correlated extensively with each other (Supplemental Table 5). In many cases very strong associations were observed (for instance, IL-12p70 associated strongly to vascular endothelial growth factor [r = 0.91, r = 0.93] and to IL-10 [r = 0.89, r = 0.92] both in women and men, respectively). Thus, in univariate analyses, multiple cytokines show correlations not only to IR, but also to each other and to several known risk factors of impaired insulin sensitivity in both sexes.

Cytokines are independent determinants of IR

To study which of the cytokines explained most of the variance in baseline IR (ie, to find a model with highest explanatory power), we used a stepwise selection method for the variable selection. The model building for contin-

Table 1. Associations of Baseline Concentrations of Cytokines to Baseline Insulin Resistance (HOMA Index)

	Women (n = 1156)		Men (n = 974)	
	Spearman r	*P*	Spearman r	*P*
Chemokines				
CCL2	0.071	.016	0.074	.021
CCL3	0.067	.022	0.055	.085
CCL4	**0.13**	**8.3 E-06**[a]	**0.19**	**2.4 E-09**
CCL11	−0.030	.31	−0.086	.0072
CCL27	**−0.26**	**7.4 E-20**	**−0.22**	**5.3 E-12**
CXCL1	0.051	.081	0.043	.18
CXCL8	0.045	.13	0.049	.12
CXCL9	0.052	.075	0.044	.17
CXCL10	0.093	.0016	**0.12**	**.0001**
CXCL12	−0.072	.014	−0.095	.0030
Growth factors				
FGF basic	0.062	.034	0.037	.25
G-CSF	0.078	.0077	−0.0038	.91
HGF	**0.24**	**7.7 E-17**	**0.27**	**2.8 E-17**
IL-7	**0.13**	**4.9 E-06**	0.060	0062
PDGF bb	**0.099**	**.0008**	0.034	.29
SCF	**0.10**	**.0006**	0.081	.012
SCGFβ	0.039	.18	0.081	.012
VEGF	**0.12**	**5.0 E-05**	0.081	.012
βNGF	0.081	.0059	0.075	.019
Other cytokines				
IFN-γ	0.074	.012	0.0021	.95
IL-1β	0.077	.0091	0.014	.67
IL-1ra	0.082	.0050	0.037	.25
IL-2	0.037	.21	−0.024	.46
IL-2rα	**0.14**	**2.7 E-06**	0.053	.099
IL-4	0.028	.34	0.019	.55
IL-5	**0.13**	**6.5 E-06**	0.076	.018
IL-6	0.050	.092	0.021	.51
IL-9	0.087	.0031	**0.11**	**0.0007**
IL-10	**0.11**	**.0003**	0.046	.15
IL-12p70	**0.098**	**.0008**	0.075	.019
IL-13	**0.13**	**1.3 E-05**	0.069	.032
IL-16	0.040	.17	−0.0083	.80
IL-17	0.022	.45	0.019	.55
IL-18	**0.23**	**2.6 E-15**	**0.24**	**9.5 E-14**
MIF	0.038	.19	**0.14**	**1.4 E-05**
TNF-α	0.028	.34	0.011	.72
TRAIL	0.037	.21	**0.12**	**.0002**

[a] Significant *P* values (after correction for multiple comparisons) are shown in bold.

uous baseline IR was done separately for both sexes and the variables available included the known risk factors (high age, BMI, triglycerides, smoking, physical inactivity, and systolic BP, low HDL cholesterol), CRP, and the 37 cytokines. The variables, which explained baseline IR, are shown in Table 2. Collectively, these variables explained 41.1% and 48.3% of the variance in IR values in women and in men, respectively. Among the cytokines, only IL-18, CCL27, and CCL11 were independent determinants for IR in both sexes. In addition, IL-4, IL-5, C-X-C motif 9, and HGF were independent determinants of IR in women, and IL-9, IL-16, macrophage migration inhibitory factor, and stem cell growth factor β in men. Thus, even when the confounding factors are taken into account, several cytokines remain as independent determinants of current IR.

Cytokines can predict the development of IR

A total of 1723 persons participated in the study both in 2007 (baseline) and 2011 (follow-up). We used the top 10% of IR (ie, the HOMA index) in each sex to define a study participant as insulin resistant. We first tested whether the baseline cytokine levels in 2007 were different in those persons who, 4 years later, had developed IR (ie, were in the 90th percentile of IR in 2011) when compared to those who had not developed IR (Table 3). In women, the levels of 16 of 37 cytokines were different according to the 90th future IR percentile. Most notably, women with IR in 2011 had 27.7% higher IL-18 and 31.3% higher HGF in 2007 than those women who did not develop IR. In men, only CCL27, CCL4, and HGF had different levels according to future IR.

To study underlying possible causal relationships, we built models to explain the future IR. The associations of cytokines with the odds of future IR (top 10% IR) were done with standardized continuous ln-transformed variables using multivariable logistic regression. The model variables were chosen from the known risk factors of IR (high age, BMI, triglycerides, smoking, physical inactivity, systolic BP, low HDL cholesterol), CRP, all cytokines, and baseline IR (year 2007) in a stepwise manner. IL-18, IL-17, BMI, and baseline IR were associated with higher odds of future IR in women (Figure 1A). In men, only BMI (OR, 1.44; 95% CI, 1.02–2.02) and baseline IR (OR, 5.15; 95% CI, 3.25–8.14) were significantly associated with higher odds of future IR.

We next analyzed whether the inclusion of cytokines would improve the overall performance of the model predicting the future development of IR compared to the standard model in women. To avoid overoptimistic predictive accuracy and validate the model building and results, we studied the performance of the model on independent test data by using 5-fold cross-validation. The standard model was built in a stepwise manner from the baseline levels of known IR risk factors (listed previously) separately in each of the five training folds. In all training folds, only baseline IR and BMI were significantly associated with the odds of future IR in a standard model (data not shown). In the new models, the 37 cytokines were available for the variable selection in addition to the known risk factors. The variables left in the new models with cytokines in each training fold are shown in Supplemental Table 6. The parameter estimates from the training folds were used to predict the outcome in the test folds (the part of the data not used in

Table 2. Independent Baseline Variables Explaining the Variance in Continuous Baseline Insulin Resistance (HOMA Index)[a]

	Parameter estimate (SE)[b]	Partial r^2	*P*
Women (n = 1100)			
ln BMI	0.32 (0.029)	0.2808	2.2E-28
ln triglycerides	0.30 (0.027)	0.0838	3.2E-27
ln HDL cholesterol	−0.082 (0.025)	0.0059	.0012
Smoking (no, yes)	−0.29 (0.069)	0.0055	3.3E-05
Physical activity index	−0.041 (0.014)	0.0056	.0040
ln IL-4	−0.076 (0.030)	0.0028	.0098
ln IL-5	0.084 (0.030)	0.0025	.0044
ln IL-18	0.092 (0.025)	0.0082	.0003
ln CCL27	−0.085 (0.025)	0.0077	.0007
ln CCL11	−0.057 (0.028)	0.0029	.041
ln CXCL9	−0.063 (0.025)	0.0026	.012
ln HGF	0.058 (0.026)	0.0027	.027
Men (n = 917)			
ln BMI	0.37 (0.029)	0.3523	1.8E-33
ln triglycerides	0.25 (0.031)	0.0779	2.8E-15
ln HDL cholesterol	−0.11 (0.028)	0.0090	.0002
ln systolic blood pressure	0.074 (0.026)	0.0048	.0041
Smoking (no, yes)	−0.20 (0.060)	0.0061	.0007
Physical activity index	−0.047 (0.013)	0.0081	.0002
ln IL-9	0.065 (0.026)	0.0030	.014
ln IL-16	−0.067 (0.026)	0.0038	.0099
ln IL-18	0.086 (0.026)	0.0047	.0013
ln CCL27	−0.073 (0.027)	0.0036	.0072
ln CCL11	−0.089 (0.026)	0.0037	.0008
ln MIF	0.070 (0.027)	0.0027	.0096
ln SCGFb	0.068 (0.025)	0.0029	.0079

[a] The stepwise linear regression model included age, BMI, CRP, triglycerides, HDL cholesterol, systolic BP, smoking, physical activity, and 37 cytokines.

[b] Parameter estimates are for 1 SD change in ln-transformed continuous variables, and one category change in categorical variables.

the model training). The pooled AUC for the standard model was 0.9034, and for the new model 0.9067, but this improvement did not reach statistical significance.

Collectively, these data indicate that in men none of the 37 cytokines was an independent predictor of future IR development. In women, in contrast, IL-18 and IL-17 showed causal relationship to the future IR in multivariable models. Interestingly, most of the known risk factors (high age, triglycerides, and systolic BP, low HDL cholesterol, smoking, or physical inactivity) or CRP did not associate with odds of future IR in the standard model, and inclusion of IL-17 and IL-18 serum concentrations in the model did not improve the predictive power of the model, implying the dominant role of baseline IR and BMI in these models.

IL-17 predicts the development of incident IR in women

Finally, we studied whether the same cytokines that were independently associated to the odds of future IR among all women would also predict development of incident IR. Future IR (top 10% IR in 2011) consisted of 42 incident cases (not in top 10% IR in 2007) and 46 prevalent cases (already in top 10% IR in 2007). Compared to the healthy subjects (not in top 10% IR in either time point), the prevalent cases as well as the incident cases had higher serum IL-17 levels already in 2007, but this was statistically significant only in the incident cases (median [25th–75th percentile] 271 [233–319] pg/ml in healthy, 302 [237–308] pg/ml in prevalent, and 306 [239–357] pg/ml in incident cases; *P* values 0.054 and 0.018, respectively). All cytokines and traditional risk factors were available for the model selection. The results showed that, in addition to the baseline IR and BMI, IL-17 remained significantly associated with the higher odds of the development of incident IR in the future (Figure 1B). Notably, in contrast to IL-17, several of the known risk factors of IR did not remain independent risk factors in prediction of incident IR. Thus, in women an increase in IL-17 levels in serum seems to precede the deterioration of insulin sensitivity.

Conclusions

We found that the circulating levels of multiple, mainly proinflammatory, cytokines are increased in those with impaired insulin sensitivity. In univariate analyses, 17 cy-

Table 3. Baseline Variables (median and 25th-75th percentile)[a] According to 4-Year Insulin Resistance (Top 10% of HOMA Index in 2011)

	Women			Men		
	Non-IR[b] (n = 821–842)	IR (n = 91–94)	*P*	Non-IR (n = 685–708)	IR (n = 75–79)	*P*
HOMA index (mU · mmol/liter^2)	1.4 (0.9–2.2)	4 (2.6–5.6)	**2.0E-34**[c]	1.6 (1–2.4)	4.3 (2.8–6.9)	**2.0E-27**
Age (y)	39 (33–42)	39 (36–42)	1.4E-01	39 (33–42)	39 (36–42)	2.8E-02
BMI (kg/m^2)	23.8 (21.5–26.5)	31 (27.5–36)	**4.1E-29**	25.7 (23.7–28.1)	30.5 (28–33.4)	**1.6E-18**
Triglycerides (mmol/liter)	0.95 (0.8–1.3)	1.5 (1.2–2.2)	**5.9E-15**	1.3 (1–1.9)	2 (1.5–2.8)	**6.3E-11**
HDL cholesterol (mmol/liter)	1.4 (1.3–1.6)	1.2 (1–1.4)	**4.8E-11**	1.2 (1–1.4)	1.1 (0.9–1.2)	**1.4E-05**
CRP (mg/liter)	0.8 (0.4–1.7)	2.6 (1–5.2)	**3.0E-15**	0.7 (0.4–1.4)	1.4 (0.8–2.9)	**8.3E-10**
Systolic BP (mm Hg)	114 (106–123)	121 (113–133)	**2.0E-07**	124 (116–133)	129 (122–135)	2.2E-03
Physical activity index	9 (8–10)	8 (7–9)	1.1E-02	9 (7–10)	8 (7–9)	3.6E-03
Insulin (mU/liter)	6.3 (3.9–9.2)	16.4 (10.9–22.2)	**1.9E-33**	6.8 (4.3–9.8)	16.7 (11.4–26.8)	**6.0E-28**
Glucose (mmol/liter)	5 (4.8–5.4)	5.6 (5.2–5.9)	**5.9E-16**	5.4 (5.1–5.7)	5.6 (5.3–6)	**5.7E-04**
Smoking (%)	13.4%	16.1%	4.3E-01	20.9%	15.4%	3.0E-01
Chemokines (pg/ml)						
CCL2	31.8 (26.3–38.9)	36.1 (28.9–46.6)	**5.5E-04**	32.9 (27—41.3)	34.8 (28.6—45.6)	1.1E–01
CCL3	12.4 (11–14.2)	13.1 (11.6–15.8)	7.4E-03	12.1 (10.8–13.8)	12.2 (10.8–14)	9.3E–01
CCL4	83.4 (65.3–102)	92.7 (75.2–111)	2.5E–03	85.6 (7–1–105)	102 (80.7–121)	**4.7E-05**
CCL11	112 (87.6–140)	115 (87.8–148)	5.3E-01	126 (96.9–161)	119 (92.5–144)	3.0E-01
CCL27	842 (686–1017)	755 (648–847)	**1.2E-05**	844 (698–1007)	712 (607–883)	**2.1E-04**
CXCL1	86.7 (60.6–115)	90.5 (74.7–141)	7.2E-03	75.9 (56.1–105)	75.2 (53.5–102)	9.6E-01
CXCL8	31.8 (28.3–35.9)	34.8 (29.2–39.8)	1.6E-03	31.1 (27.8–34.8)	31.8 (28.3–36.5)	2.5E-01
CXCL9	442 (344–602)	498 (367–744)	9.1E-03	440 (344–599)	475 (351–639)	2.5E-01
CXCL10	585 (442–808)	707 (518–938)	**1.7E-04**	588 (439–813)	676 (489–993)	7.3E-03
CXCL12	69.6 (50.7—90.4)	64.5 (51–86.9)	3.3E–01	62.4 (44–79.5)	53.1 (31.1–76)	1.4E-02
Growth factors (pg/ml)						
FGF basic	67.6 (58.1–81.7)	73.8 (58.2–92)	3.4E-02	65.9 (56.5–76.6)	66.9 (58.8–82)	4.3E-01
G-CSF	138 (119–164)	154 (130–175)	1.1E-03	134 (117–155)	137 (119–153)	7.2E-01
HGF	501 (402–652)	658 (533–838)	**3.0E-11**	491 (395–603)	588 (475–724)	**3.8E-07**
IL-7	20.6 (16.8–25.1)	23.8 (20.3–28.5)	**3.5E-05**	19.7 (16.2–23.8)	20.1 (17.4–25.2)	9.1E-02
PDGF bb	8468 (6759–10 190)	9429 (7761–11 415)	2.9E-03	8640 (6973–10 574)	8767 (6870–11 578)	4.9E-01
SCF	85.7 (68.4–103)	89.7 (75.5–113)	1.6E-02	97.6 (80–113)	99.2 (77.3–121)	8.3E-01
SCGFβ	10 293 (8153–13 262)	11 235 (8041–13 681)	2.9E-01	11 616 (9109–14 676)	12 636 (9460–16 010)	8.8E-02
VEGF	70.6 (50.6–103)	101 (62.3–133)	**7.7E-05**	71.3 (47.7–104)	76.3 (55.8–116)	1.0E-01
βNGF	1.4 (1.1–1.8)	1.6 (1.3–2)	**4.6E-05**	1.3 (1–1.7)	1.4 (1–1.9)	5.5E-01
Other cytokines (pg/ml)						
IFN-γ	267 (228–313)	283 (248–344)	6.2E-03	259 (224–306)	258 (223–303)	8.8E-01
IL-1β	4.9 (4.2–5.8)	5.4 (4.5–6.8)	**2.4E-04**	4.8 (4.1–5.5)	4.6 (4–5.6)	8.8E-01
IL-1ra	236 (194–284)	260 (209–324)	4.9E-03	219 (187–266)	230 (197–284)	2.6E-01
IL-2	19.2 (16.1–22.3)	20 (17.4–24.2)	2.5E-02	18.4 (16–21.2)	18.3 (15.2–20.8)	5.9E-01
IL-2rα	72.8 (52.1–94.8)	89.5 (65.3–120)	**3.7E-05**	81.2 (61.7–104)	86 (58.1–116)	1.5E-01
IL-4	11.5 (10.4–12.6)	12.1 (10.8–13.6)	4.3E-03	11.4 (10.3–12.5)	11.2 (10.3–12.3)	9.0E-01
IL-5	6 (5.1–7.2)	6.8 (5.7–8.2)	**1.3E-04**	5.9 (5.1–6.9)	6.2 (5–7)	7.8E-01
Il-6	12 (10.2–13.9)	13.3 (10.8–15.6)	2.2E-03	11.4 (10–13.1)	11.4 (9.8–14)	6.0E-01
IL-9	57.2 (46.2–75.8)	63 (51.4–97.2)	1.1E-02	53.7 (43.8–66.6)	58.2 (46.8–81.4)	5.1E-02
IL-10	19.3 (13.8–24.8)	23.4 (18.7–30.4)	**3.1E-05**	18.3 (12.9–24.5)	18.7 (13.4–25.5)	3.3E-01
IL-12p70	68.5 (48.2–91)	85.9 (61.5–114)	**5.8E-05**	65.5 (45.7–90.7)	68 (51.8–99.3)	1.2E-01
IL-13	17.5 (13.9–21.3)	20.2 (16.2–25.7)	**2.2E-05**	17.5 (13.5–21.5)	18.8 (14.8–23.6)	4.4E-02
IL-16	71.5 (40.4–103)	81 (54–111)	2.0E-02	76.2 (44.6–108)	74.4 (47.8–96.1)	7.9E-01
IL-17	270 (233–319)	304 (237–354)	2.8E-03	264 (229–306)	260 (221–319)	7.5E-01
IL-18	60.2 (44.5–75.8)	76.9 (55.9–105)	**8.7E-09**	69.9 (53.7–90.6)	82.8 (58.7–105)	5.6E-03
MIF	151 (109–213)	180 (115–233)	1.0E-01	163 (107–227)	199 (116–248)	1.6E-02
TNF-α	48.7 (40.5–59.3)	54.4 (45.6–65.7)	**6.1E-04**	48.6 (41.1–56.2)	48.5 (41–55.4)	9.8E-01
TRAIL	111 (86.3–143)	135 (94–175)	**4.0E-04**	154 (121–192)	167 (127–230)	1.5E-02

Abbreviations: CXC, C-X-C motif; G-CSF, granulocyte colony-stimulating factor; IR, insulin resistance; NGF, nerve growth factor; PDGF, platelet-derived growth factor; SCGF, stem cell growth factor; TRAIL, tumor necrosis factor-related apoptosis inducing ligand; VEGF, vascular endothelial growth factor.

[a] Except for smoking, which is presented as %.

[b] Significant *P* values (after correction for multiple comparisons) are shown in bold.

tokines were associated with IR. The levels of many of these same cytokines increased along with the increasing BMI. Nevertheless, in multivariable regression analyses, fully adjusted for BMI and other known risk factors of IR, the proinflammatory T helper 1 cell (Th1)-type cytokine IL-18 still showed positive and T helper 2 cell-type cytokine CCL11 negative associations with IR in both sexes. Most importantly, circulating proinflammatory cytokines (T helper 17 cell [Th17]-type IL-17 and IL-18) were predictive biomarkers for future development of IR in models

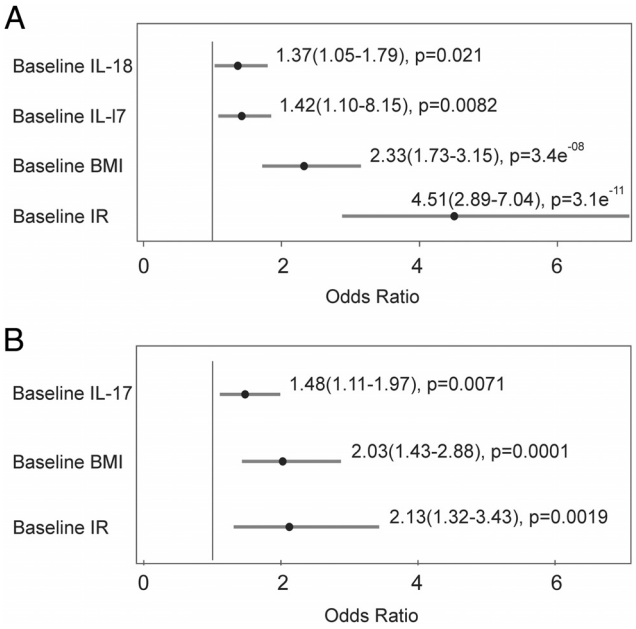

Figure 1. Adjusted odds ratios of baseline variables for future insulin resistance (IR). A, Adjusted ORs (with 95% confidence intervals) for future IR when both incident and prevalent IR cases are as the outcome. B, Adjusted ORs (with 95% confidence limits) for future IR when only incident IR cases are as the outcome. Black circle = odds ratio (OR); gray line = 95% confidence limits of the OR.

fully adjusted for baseline IR and other known risk factors of IR in women. IL-17 also predicted the development of future IR 4 years later among those women who did not have IR at the time of analyses. In contrast, general low-grade inflammation reflected as increased CRP level was neither explanatory nor predictive biomarker for IR. Our data thus suggest that specific IL-17-driven inflammation may precede the deterioration of insulin sensitivity in general population.

We found that cytokines IL-17 and IL-18 were predictive markers for future IR in multivariable regression models in women. IL-17 in particular had relatively high OR (1.42) in predicting 4-year IR in women. Interestingly, human liver and skeletal muscles synthesize receptors for IL-17. Adipose tissue of obese subjects contains increased numbers of Th17 cells in comparison to lean subjects, and administration of IL-17 reduces insulin sensitivity in cultured human liver cells (20). Th17 cells are increased in the blood of type 2 diabetes patients (21), and standard treatment of newly diagnosed type 2 diabetes reduces IL-17 levels in blood (22). In genetic mouse models, IL-17 is causally involved in exacerbating diet-induced obesity and adipose tissue deposition, and anti-IL-17 treatment enhances glucose uptake in skeletal muscle (23, 24). However, in humans, the association of IL-17 with glucose tolerance is not firmly established because others have reported decreased levels of IL-17 in metabolic syndrome (25). Our data with cross-validated predictive models strongly support the notion that Th17 type inflammation precedes and may be pathologically relevant to the development of IR.

High CRP has been suggested to be a marker of emerging IR (26–30). We found that CRP levels are indeed significantly higher in persons with IR and in obese persons, and that CRP positively correlates with many proinflammatory cytokines. However, CRP did not remain as an independent contributor for current or developing IR in our study cohort. We thus hypothesize that the general low-level inflammation reflected by increased CRP values is not relevant for the development of IR, but instead, proinflammatory Th17-dominant subclinical inflammation is more important in the impairment of insulin sensitivity.

Many of the correlations between cytokines and IR disappeared when BMI was taken into account as a confounding factor. These results support the notion that the elevations in systemic cytokines in IR are often merely secondary to the increased fat cell mass, and that the cytokines can be produced by the inflamed fat tissue. We also found significant differences in the levels of half of the measured cytokines between females and males. Although the differences between the sexes in the absolute concentrations of these cytokines were often modest, it is notable that the cytokines associated to or predictive of IR were very different among women and men. The reason for the marked sex-dependent variations in cytokine levels remains speculative. However, it is firmly established that sex steroids heavily affect both the nature and extent of immune responses. For instance, estrogen increases IL-17 and decreases HGF production in rodent models (31, 32). Notably, we also found that in the regression model (eg, IL-17 *), the gender interaction term was statistically significant. Our findings thus strongly implicate that all association studies between cytokines and IR have to be done and interpreted in a sex-specific manner and by adjusting for BMI or another measure of obesity, which often has not been the case in previous studies.

We are aware that our study has certain limitations. Because oral glucose tests were not included in the study protocol, we had to use the HOMA index as an approximation of insulin sensitivity. It is known that the impaired stimulated glucose tolerance is the most sensitive measure of developing IR. Nevertheless, in a relatively young population, such as in our cohort, HOMA index is still regarded as a relatively reliable marker of IR (33). We feel that the 90th percentile cutoff separately for each gender is useful for finding the insulin resistant individuals. We did not have available a replication cohort to study the predictive value of the cytokines. How-

ever, we used rigorous 5-fold cross validations in our reclassification analyses to control for the potential overfitting of the models. Finally, our study was performed in an ethnically homogenous European population, and therefore the validity of the results in other populations remains to be confirmed.

In conclusion, we found that baseline IR, BMI, IL-18, and IL-17, but not other traditional risk factors, other cytokines or CRP, predicted the development of future IR in women in multivariable models. The specific proinflammatory cytokine IL-17 was also an independent risk factor for incident IR in women.

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Address all correspondence and requests for reprints to: Marko Salmi, MediCity Research Laboratory, Tykistökatu 6A, 20520 Turku, Finland. E-mail: marko.salmi@utu.fi.

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References

1. **Donath MY, Shoelson SE.** Type 2 diabetes as an inflammatory disease. *Nat Rev Immunol*. 2011;11:98–107.
2. **Kahn SE, Hull RL, Utzschneider KM.** Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature*. 2006;444:840–846.
3. **Uysal KT, Wiesbrock SM, Marino MW, Hotamisligil GS.** Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. *Nature*. 1997;389:610–614.
4. **McGillicuddy FC, Harford KA, Reynolds CM, Oliver E, Claessens M, Mills KH, Roche HM.** Lack of interleukin-1 receptor I (IL-1RI) protects mice from high-fat diet-induced adipose tissue inflammation coincident with improved glucose homeostasis. *Diabetes*. 2011;60:1688–1698.
5. **Aguirre V, Uchida T, Yenush L, Davis R, White MF.** The c-Jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser(307). *J Biol Chem*. 2000;275:9047–9054.
6. **He J, Usui I, Ishizuka K, et al.** Interleukin-1alpha inhibits insulin signaling with phosphorylating insulin receptor substrate-1 on serine residues in 3T3–L1 adipocytes. *Mol Endocrinol*. 2006;20:114–124.
7. **Shoelson SE, Lee J, Goldfine AB.** Inflammation and insulin resistance. *J Clin Invest*. 2006;116:1793–1801.
8. **Donath MY.** Targeting inflammation in the treatment of type 2 diabetes: time to start. *Nat Rev Drug Discov*. 2014;13:465–476.
9. **Bernstein LE, Berry J, Kim S, Canavan B, Grinspoon SK.** Effects of etanercept in patients with the metabolic syndrome. *Arch Intern Med*. 2006;166:902–908.
10. **Paquot N, Castillo MJ, Lefebvre PJ, Scheen AJ.** No increased insulin sensitivity after a single intravenous administration of a recombinant human tumor necrosis factor receptor: Fc fusion protein in obese insulin-resistant patients. *J Clin Endocrinol Metab*. 2000;85:1316–1319.
11. **Wascher TC, Lindeman JH, Sourij H, Kooistra T, Pacini G, Roden M.** Chronic TNF-alpha neutralization does not improve insulin resistance or endothelial function in "healthy" men with metabolic syndrome. *Mol Med*. 2011;17:189–193.
12. **Larsen CM, Faulenbach M, Vaag A, Ehses JA, Donath MY, Mandrup-Poulsen T.** Sustained effects of interleukin-1 receptor antagonist treatment in type 2 diabetes. *Diabetes Care*. 2009;32:1663–1668.
13. **Larsen CM, Faulenbach M, Vaag A, et al.** Interleukin-1-receptor antagonist in type 2 diabetes mellitus. *N Engl J Med*. 2007;356:1517–1526.
14. **Makki K, Froguel P, Wolowczuk I.** Adipose tissue in obesity-related inflammation and insulin resistance: cells, cytokines, and chemokines. *ISRN Inflamm*. 2013;2013:139239.
15. **Osborn O, Olefsky JM.** The cellular and signaling networks linking the immune system and metabolism in disease. *Nat Med*. 2012;18:363–374.
16. **Juonala M, Viikari JS, Raitakari OT.** Main findings from the prospective Cardiovascular Risk in Young Finns Study. *Curr Opin Lipidol*. 2013;24:57–64.
17. **Raitakari OT, Juonala M, Ronnemaa T, et al.** Cohort profile: the cardiovascular risk in Young Finns Study. *Int J Epidemiol*. 2008;37:1220–1226.
18. **Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC.** Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia*. 1985;28:412–419.
19. **Gupta V, Zhou H, Reyes C, Wang Q.** Multiplexing across cytokine panels: Bio-Plex Pro Human and Mouse Group I and Group II. 2010; http://www.bio-rad.com/webroot/web/pdf/lsr/literature/bulletin_5927.pdf. Accessed 9 March, 2016.
20. **Fabbrini E, Cella M, McCartney SA, et al.** Association between specific adipose tissue CD4+ T-cell populations and insulin resistance in obese individuals. *Gastroenterology*. 2013;145:366–374 e361–363.
21. **Jagannathan-Bogdan M, McDonnell ME, Shin H, et al.** Elevated proinflammatory cytokine production by a skewed T cell compartment requires monocytes and promotes inflammation in type 2 diabetes. *J Immunol*. 2011;186:1162–1172.
22. **Sumarac-Dumanovic M, Jeremic D, Pantovic A, et al.** Therapeutic improvement of glucoregulation in newly diagnosed type 2 diabetes patients is associated with a reduction of IL-17 levels. *Immunobiology*. 2013;218:1113–1118.
23. **Zuniga LA, Shen WJ, Joyce-Shaikh B, et al.** IL-17 regulates adipogenesis, glucose homeostasis, and obesity. *J Immunol*. 2010;185:6947–6959.
24. **Ohshima K, Mogi M, Jing F, et al.** Roles of interleukin 17 in angiotensin II type 1 receptor-mediated insulin resistance. *Hypertension*. 2012;59:493–499.
25. **Surendar J, Aravindhan V, Rao MM, Ganesan A, Mohan V.** Decreased serum interleukin-17 and increased transforming growth factor-beta levels in subjects with metabolic syndrome (Chennai Urban Rural Epidemiology Study-95). *Metabolism*. 2011;60:586–590.
26. **Fang P, Shi M, Yu M, Guo L, Bo P, Zhang Z.** Endogenous peptides

as risk markers to assess the development of insulin resistance. *Peptides*. 2014;51:9–14.
27. **Ford ES.** Body mass index, diabetes, and C-reactive protein among U.S. adults. *Diabetes Care*. 1999;22:1971–1977.
28. **Bremer AA, Jialal I.** Adipose tissue dysfunction in nascent metabolic syndrome. *J Obes*. 2013;2013:393192.
29. **Timpson NJ, Lawlor DA, Harbord RM, et al.** C-reactive protein and its role in metabolic syndrome: mendelian randomisation study. *Lancet*. 2005;366:1954–1959.
30. **Taniguchi A, Nagasaka S, Fukushima M, et al.** C-reactive protein and insulin resistance in non-obese Japanese type 2 diabetic patients. *Metabolism*. 2002;51:1578–1581.
31. **Khan D, Dai R, Karpuzoglu E, Ahmed SA.** Estrogen increases, whereas IL-27 and IFN-gamma decrease, splenocyte IL-17 production in WT mice. *Eur J Immunol*. 2010;40:2549–2556.
32. **Wang YC, Xu GL, Jia WD, et al.** Estrogen suppresses metastasis in rat hepatocellular carcinoma through decreasing interleukin-6 and hepatocyte growth factor expression. *Inflammation*. 2012;35:143–149.
33. **Bonora E, Targher G, Alberiche M, et al.** Homeostasis model assessment closely mirrors the glucose clamp technique in the assessment of insulin sensitivity: studies in subjects with various degrees of glucose tolerance and insulin sensitivity. *Diabetes Care*. 2000;23:57–63.