Published in final edited form as:

Atherosclerosis. 2011 December ; 219(2): 869–874. doi:10.1016/j.atherosclerosis.2011.09.005.

Free Fatty Acids Are Associated with Insulin Resistance but Not Coronary Artery Atherosclerosis in Rheumatoid Arthritis

Michelle J Ormseth^a, Larry L Swift^a, Sergio Fazio^a, MacRae F Linton^a, Cecilia P Chung^a, Paolo Raggi^b, Young Hee Rho^a, Joseph Solus^c, Annette Oeser^a, Aihua Bian^d, Tebeb Gebretsadik^d, Ayumi Shintani^d, and C. Michael Stein^a

^aDivisions of Rheumatology, Clinical Pharmacology; Departments of Medicine and Pharmacology, Vanderbilt University, Nashville, Tennessee

^bDivision of Cardiology, Emory University, Atlanta, Georgia

^cDepartment of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, Tennessee

^dDepartment of Biostatistics, School of Medicine, Vanderbilt University, Nashville, Tennessee

Abstract

Background—Free fatty acids (FFAs) affect insulin signaling and are implicated in the pathogenesis of insulin resistance and atherosclerosis. Inflammatory cytokines such as interleukin-6 (IL-6) increase lipolysis and thus levels of FFAs. We hypothesized that increased IL-6 concentrations are associated with increased FFAs resulting in insulin resistance and atherosclerosis in rheumatoid arthritis (RA).

Methods—Clinical variables, serum FFAs and inflammatory cytokines, homeostasis model assessment for insulin resistance (HOMA-IR), and coronary artery calcium were measured in 166 patients with RA and 92 controls. We compared serum FFAs in RA and controls using Wilcoxon rank sum tests and further tested for multivariable association by adjusting for age, race, sex and BMI. Among patients with RA, we assessed the relationship between serum FFAs and inflammatory cytokines, HOMA-IR, and coronary artery calcium scores using Spearman correlation and multivariable regression analysis.

Results—Serum FFAs did not differ significantly in patients with RA and controls (0.56 mmol/L [0.38–0.75] and 0.56 mmol/L [0.45–0.70] respectively, $p=0.75$). Presence of metabolic syndrome was associated with significantly increased serum FFAs in both RA and controls ($p=0.035$ and $p=0.025$). In multivariable regression analysis that adjusted for age, race, sex and BMI, serum FFAs were associated with HOMA-IR ($p=0.011$), CRP ($p=0.01$), triglycerides ($p=0.005$) and Framingham risk score ($p=0.048$) in RA, but not with IL-6 ($p=0.48$) or coronary artery calcium score ($p=0.62$).

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Correspondence to: Michelle J Ormseth, MD, 1161 21st Avenue South, T-3219 Medical Center North, Nashville, TN 37232-2681, USA, michelle.ormseth@vanderbilt.edu, Tel: 1-615-322-4746, FAX: 1-615-322-6248.

Disclosures: None

Conflict of Interest: There was no conflict of interest.

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Conclusions—Serum FFAs do not differ significantly in patients with RA and controls. FFAs may contribute to insulin resistance, but are not associated with IL-6 and coronary atherosclerosis in RA.

Introduction

Patients with rheumatoid arthritis (RA) have an increased prevalence of premature atherosclerosis even after adjusting for traditional cardiovascular risk factors.¹ We have reported that patients with RA have an increased prevalence of insulin resistance which is associated with coronary atherosclerosis,² but the underlying mechanisms are unclear. Free fatty acids (FFAs) have been implicated as a mechanistic explanation for the relationship between obesity, increased inflammation, insulin resistance and cardiovascular disease.³

FFAs are released from adipocytes through lipolysis, and because of the relative increase in adipose tissue, plasma FFAs are elevated in obesity.⁴ Insulin down-regulates lipolysis and under conditions of insulin resistance this regulatory mechanism is impaired, resulting in increased FFAs.^{5,6} Moreover, elevated plasma FFAs cause peripheral insulin resistance, promoting an additional increase in release of FFAs because the anti-lipolytic action of insulin is impaired.⁷

Elevated FFAs result in decreased endothelial nitric oxide production and increased reactive oxygen species in cultured vascular endothelial cells.⁸ Thus, high concentrations of FFAs promote endothelial dysfunction,⁹ a potential mechanism underlying atherosclerosis and coronary artery disease. In addition to promoting insulin resistance, endothelial dysfunction and atherosclerosis, FFAs appear to both increase inflammation and be increased by inflammation.

Acute elevation of FFAs increases hepatic expression of interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF- α) in a rat model given a lipid/heparin infusion.¹⁰ In healthy humans an acute increase in FFAs via lipid/heparin infusion also induces inflammatory changes and oxidative stress, including NF- κ B binding activity, p65 expression in circulating mononuclear cells, and generation of reactive oxygen species in mononuclear and polymorphonuclear leukocytes.¹¹ FFAs, in addition to promoting inflammation, are also increased by inflammation. Serum amyloid A (SAA) and key inflammatory cytokines such as IL-6 and TNF- α stimulate lipolysis and increase FFAs.^{12,13,14}

These observations raise the question whether FFAs play a role in the inflammation, insulin resistance and atherosclerosis associated with RA, a disease characterized by increased chronic inflammation. We therefore addressed the previously unexplored hypothesis that increased IL-6 concentrations are associated with increased FFAs, resulting in insulin resistance and atherosclerosis in RA.

Materials and Methods

Study population

We studied 166 subjects with RA and 92 control subjects frequency matched for age, race and sex that comprise a cohort in whom we have defined the relationships between inflammation and atherosclerosis.^{1,2,21,26} Recruitment and study procedures have been described in detail.¹ Subjects were older than 18 years of age and patients with RA fulfilled the classification criteria for RA.¹⁵ Control subjects did not have RA or other inflammatory disease. The study was approved by the Vanderbilt University Institutional Review Board and all subjects gave written informed consent.

Clinical Data

Clinical information, laboratory data, and coronary calcium scores were obtained as described previously.¹ FFAs were measured from serum samples obtained after an overnight fast using an enzymatic assay (HR series NEFA-HR(2) assay, Wako Diagnostics, Richmond, VA, USA) and expressed as mmol/L. We used a Shimadzu UV1201 UV/Vis spectrophotometer for measurement of optical densities. The expected normal range for serum FFAs for fasting patients is 0.1-0.6 mmol/L. The range of linearity of this method is up to 2.0 mmol/L. The inter-assay coefficient of variation is 2.7% for normal levels and 1.1% for elevated levels. Samples were stored at -80°C for up to 10 years before that analysis. The samples were run together in batches. Coronary artery calcium was measured by electron beam computed tomography (EBCT) scanning with an Imatron C-150 scanner (GE/Imatron, South San Francisco, CA, USA) and was quantified in Agatston units.¹⁶ Disease activity of rheumatoid arthritis was determined through the 28 joint count disease activity score (DAS28).¹⁷ The Framingham risk score was calculated using blood pressure, smoking status, serum lipid concentrations, age and sex, but not diabetes.¹⁸ Body mass index (BMI) was calculated and expressed as kg/m^2 . Insulin resistance was measured using the homeostasis model assessment of insulin resistance (HOMA-IR) index and calculated as $[\text{serum insulin } (\mu\text{U/ml}) \times \text{glucose (mmol/l)}] / 22.5$.¹⁹ Glucose, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), total cholesterol, triglycerides, high density lipoprotein (HDL) and low density lipoprotein (LDL) were measured by the Vanderbilt University Medical Center Clinical Laboratory. Before 2003, the laboratory did not use a high-sensitivity CRP assay, and low concentrations were reported as $< 3 \text{ mg/L}$. Thus, in 40 patients with CRP reported as $< 3 \text{ mg/L}$, concentrations were measured by ELISA (Millipore). TNF- α , IL-6, SAA, and insulin were measured by multiplex ELISA (Lincoplex[®] Multiplex Immunoassay Kit, Millipore Corp., Billerica MA, USA). A patient was defined as having metabolic syndrome, in accordance with the 2005 Adult Treatment Panel (ATP) III criteria, based on the presence of 3 or more of the following 5 characteristics: abdominal obesity (waist circumference $> 102 \text{ cm}$ in men and $> 88 \text{ cm}$ in women), serum triglycerides $\geq 150 \text{ mg/dL}$ or on drug treatment for hypertriglyceridemia, serum HDL $< 40 \text{ mg/dL}$ in men or $< 50 \text{ mg/dL}$ in women or on drug treatment for low HDL, blood pressure $\geq 130/85 \text{ mmHg}$ or on antihypertensive therapy, and fasting plasma glucose $\geq 100 \text{ mg/dL}$, or on treatment for elevated blood sugar.²⁰

Statistical Analysis

Descriptive statistics were calculated as median with the interquartile range (median [IQR]) for continuous variables, and frequency and proportion for categorical variables. We used Wilcoxon's rank sum test to compare continuous variables between RA patients and control subjects, and Pearson's chi-square test to compare categorical variables. To assess the correlation between serum FFAs and continuous clinical variables including the coronary artery calcium score, Spearman's rank correlation coefficients (ρ) was calculated separately among patients with RA and control subjects.

The independent associations between serum FFAs and components of the lipid profile (total cholesterol, LDL, HDL and triglycerides), markers of insulin resistance (HOMA-IR, fasting glucose and insulin), inflammation (CRP, SAA, IL-6, TNF- α , and ESR), Framingham risk score, current use of medications, and RA disease activity (DAS28 score) were assessed using multivariable linear regression models with adjustment for age, race, sex and BMI.

The association of serum FFAs with coronary artery calcium score was examined with a proportional odds logistic regression model adjusting for age, race, sex and Framingham risk score. Concentrations of serum FFAs, triglycerides, HOMA-IR, CRP, TNF- α , IL-6 were

natural logarithm-transformed to improved normality. Statistical analyses were performed using R version 2.10.0 (<http://www.r-project.org>) and 2-sided P values less than 0.05 were considered statistically significant.

Results

Demographic and clinical and laboratory characteristics of RA and control cohorts have been reported previously (Table 1).^{1,2} Patient and control groups were similar with regard to age, gender, race and BMI. Markers of inflammation, including CRP, SAA, IL-6, and TNF- α were all higher in RA patients (all $p < 0.001$).²¹ As previously reported in this cohort,^{1,2,22} fasting insulin concentrations were higher in RA than controls (10.51 uIU/ml [5.29-17.09] vs. 3.77 uIU/ml [2.46-7.99] $p < 0.001$) (Figure 1), as was insulin resistance as measured by HOMA-IR (2.34 [1.16-4.27] vs. 0.825 [0.535-1.785], $p < 0.001$), and coronary calcium score (2.7 [0.0-150.4] vs. 0.0 [0.0-18.7], $p = 0.016$).

Concentrations of serum FFAs in patients with RA and controls were similar (0.56 mmol/L [0.383-0.748] vs 0.56 mmol/L [0.45-0.7], $p = 0.745$) (Figure 2). After adjustment for age, race, sex and BMI there was no statistical evidence for association between disease status and serum FFAs ($p = 0.143$). Serum FFAs were positively correlated with BMI in both RA and control subjects ($\rho = 0.155$, $p = 0.046$; $\rho = 0.2$, $p = 0.006$), but not with age in either RA ($\rho = 0.066$, $p = 0.396$) or controls ($\rho = 0.156$, $p = 0.136$).

In patients with RA and controls, serum FFAs were correlated with HOMA-IR ($\rho = 0.198$, $p = 0.011$ and $\rho = 0.253$, $p = 0.016$, respectively); these associations remained significant after adjustment for age, sex, race and BMI (Table 2 and Figure 3). Serum FFAs were higher in RA and control subjects with metabolic syndrome compared to those without (0.62 mmol/L [0.44-0.80] vs. 0.54 mmol/L [0.37-0.69]), $p = 0.035$; and 0.63 mmol/L [0.55-0.76] vs 0.54 mmol/L [0.45-0.66], $p = 0.025$, respectively) (Figure 4).

Serum FFAs were correlated with CRP ($\rho = 0.253$, $p = 0.001$). Similarly, serum FFAs were correlated with SAA ($\rho = 0.185$, $p = 0.017$), but this association was attenuated after adjustment ($p = 0.081$). There was no association with serum FFAs and IL-6 or TNF- α in patients with RA (Table 2). In controls serum FFAs were correlated with CRP ($\rho = 0.255$, $p = 0.015$), but this association was not significant after adjustment ($p = 0.218$) (Table 2).

In patients with RA serum FFAs were positively correlated with cardiovascular risk as determined by the Framingham risk score ($\rho = 0.215$, $p = 0.005$) (Table 2). The association remained significant after adjustment for age, sex, race and BMI ($p = 0.048$). In control subjects serum FFAs were correlated with the Framingham risk score ($\rho = 0.281$, $p = 0.007$), but this was not significant after adjustment similar covariates. Coronary artery calcium was not associated with serum FFAs in both univariate analysis and in multivariable analysis after adjustment for covariates in both RA and control subjects (Table 2).

Serum FFAs did not differ significantly in patients with RA currently using or not using hydroxychloroquine, methotrexate, prednisone or anti-TNF agents (all p values ≥ 0.6) (Table 3). Similarly, statin use was not associated with serum FFAs ($p = 0.4$).

Discussion

The major new finding of this study is that serum FFAs may contribute to insulin resistance in patients with RA, but are not associated with IL-6 concentration or coronary calcification. Moreover, serum FFAs were not higher in patients with RA as compared to controls, despite the presence of increased inflammation in RA.

Elevation of FFAs can increase inflammation, and particularly relevant to RA, inflammatory cytokines can increase FFAs. Several *in vitro* studies found that adding IL-6 and TNF- α to isolated adipocytes resulted in increased lipolysis.^{23,24} Furthermore, infusions of IL-6 or TNF- α in healthy subjects resulted in acutely increased circulating FFAs.^{25,26} However, these studies examined acute changes. While it might be considered a limitation of the study that the RA patients were receiving treatment for their disease, potentially altering results, RA patients reflect the clinical situation in which increased atherosclerosis occurs. Furthermore, despite treatment, they had significantly elevated inflammatory markers compared to controls, providing the opportunity to detect an inflammation-related signal. In our study, RA patients were chronically exposed to increased IL-6 and TNF- α , and there was no correlation between these cytokines and serum FFAs. There was, however, a significant correlation between serum FFAs and CRP.

It is not clear why serum FFAs were associated with CRP but not IL-6 or TNF- α in RA. IL-6 is considered to be the major regulator of CRP synthesis in the liver; however, CRP and IL-6 are not always co-regulated.²⁷ For example, weight loss has been associated with a decrease in CRP, but no change in IL-6.²⁸ Similarly, in the Women's Health Initiative study hormone replacement therapy was associated with increased CRP but not IL-6.²⁹ Thus, it is possible that in RA the relationship between FFAs and IL-6, and FFAs and CRP could differ.

Several lines of evidence suggest that circulating FFAs are associated with insulin resistance and that there may be a cause and effect relationship. For example, insulin resistance developed when plasma FFAs were increased in healthy subjects through lipid infusion.³⁰ Conversely, decreasing the chronically elevated plasma FFAs of obese patients with acipimox, a long acting antilipolytic drug, improved insulin sensitivity.³¹ We found that in RA, as was the case in controls, serum FFAs correlated with insulin resistance. This correlation was relatively weak, but present in both RA and controls, suggesting that the inflammatory milieu of RA was not a prerequisite. Similarly, both RA and control subjects with metabolic syndrome, an insulin resistant state, had higher serum FFA concentrations. Because insulin resistance may be mechanistically associated with atherosclerosis, we evaluated if serum FFAs were associated also with the presence of coronary calcium.

The evidence linking FFAs and atherosclerosis is based in part on their known effects on vascular endothelium and smooth muscle.³² For example, infusing a lipid emulsion and increasing circulating FFAs to levels seen in insulin resistance resulted in a decrease in endothelium-dependent vasodilation.³³ Also, a transient increase in plasma FFAs to the range seen in obesity and type 2 diabetes mellitus induced markers of endothelial activation, vascular inflammation and thrombosis in healthy subjects.³⁴ In addition to these effects, elevation of some FFAs promotes uptake of oxidized LDL in macrophages, a critical step in development of atherosclerosis.³⁵

Additional evidence links FFAs with atherosclerosis. A Japanese study comparing type 2 diabetic patients with age and sex-matched, non-diabetic controls found that serum FFAs were strongly correlated with carotid intimal media thickness (IMT) in diabetics, but not control subjects.³⁶ Similarly, there was a significant association between serum FFAs and carotid IMT in renal transplant recipients, a population in whom FFAs are elevated and in whom chronic inflammation and insulin resistance are present.³⁷ However, in our study serum FFAs were not associated with coronary artery calcium score in either RA or control subjects.

Our study had some limitations such as the cross-sectional design that precludes the inference of a cause and effect relationship with regard to FFAs and insulin resistance. Also,

we needed to use two different assays for CRP measurement. One suitable for low CRP concentrations, as occurs in controls and patients without much inflammation, and another suitable for high CRP concentrations, as occurs in patients with active inflammation. We cannot exclude the possibility that serum FFAs may be related to non-calcified coronary atherosclerotic plaque, because we only measured calcified lesions. Additionally, we only studied fasting circulating FFAs, and not the rate of appearance, postprandial concentrations, intracellular concentrations, or portal levels of FFAs³⁸; specific studies to address those responses in patients with RA and controls will be of interest.

In conclusion, concentrations of serum FFAs do not differ significantly in patients with RA and controls. FFAs may contribute to insulin resistance in RA as they do in controls, but are not associated with IL-6 and coronary atherosclerosis in RA. The contribution of FFAs to atherogenesis and insulin resistance may be independent of each other and may vary in different diseases.

Acknowledgments

Funding: This study was supported by grant P60 AR056116.

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Figure 1. Plasma insulin concentration (uIU/ml) in RA and control subjects. Plasma insulin concentrations were significantly greater in RA than controls (10.59 uIU/ml [5.28-18.04] and 3.78 uIU/ml [2.45-8.11], $p < 0.001$).

Figure 2. Serum free fatty acid concentration (mmol/L) in RA (n=166) and control subjects (n=92). There was no difference between the RA and control patients 0.56 mmol/L [0.383-0.748] vs 0.56 mmol.L [0.45-0.7], $p=0.745$.

Figure 3.

Relationship between serum FFAs and HOMA-IR in RA patients. Data is presented on a logarithmic scale. HOMA-IR was correlated with FFAs ($Rho=0.198$, $p=0.011$, adjusted for age, race, sex and BMI $p=0.013$). HOMA-IR= Homeostasis Model Assessment of Insulin Resistance.

Figure 4.

Serum free fatty acids in RA and control subjects with and without metabolic syndrome. FFAs were higher in RA and control subjects with metabolic syndrome compared to those without (0.62mmol/L [0.44-0.80] vs. 0.54 mmol/L [0.37-0.69], $p=0.035$; and 0.63 mmol/L [0.55-0.76] vs 0.54 mmol/L [0.45-0.66], $p=0.025$, respectively).

Table 1
Characteristics of RA and control subjects

	RA N = 166	Controls N = 92	P value
Age (Year)	54.0 [45.0-62.8]	53.0 [44.8-59.2]	0.442
Sex (% Male)	31%	37%	0.358
Race (% Caucasian)	89%	85%	0.307
BMI (kg/m ²)	28.34 [23.99-33.2]	27.01 [24.59-31.81]	0.451
DAS28 Score	3.88 [2.64-4.84]	-	-
Current Smoker	24%	9%	0.002
Hypertension	52%	39%	0.041
Systolic Blood Pressure (mmHg)	132.8 [117.5-145.5]	128.5 [114.9-136.8]	0.09
Diastolic Blood Pressure (mmHg)	74.75 [67.12-82.0]	72.25 [67.5-78.0]	0.135
Diabetes	11%	4%	0.055
Metabolic Syndrome	36%	22%	0.02
HOMA-IR	2.34 [1.16-4.27]	0.825 [0.535-1.785]	<0.001
Fasting glucose (mg/dL)	87.0 [83.0-94.8]	89.0 [83.0-94.2]	0.801
Insulin (uIU/ml)	10.51 [5.29-17.09]	3.77 [2.46-7.99]	<0.001
Statin Use	13%	13%	0.928
Total cholesterol (mg/dL)	184.0 [156.0-211.0]	195.0 [170.0-216.0]	0.075
HDL cholesterol (mg/dL)	43.0 [37.0-54.0]	45.0 [38.5-54.0]	0.533
LDL cholesterol (mg/dL)	111.0 [88.0-135.0]	122.0 [104.0-145.0]	0.016
Triglycerides (mg/dL)	112.0 [80.0-158.0]	103.0 [73.0-135.5]	0.175
CRP (mg/L)	4.00 [1.198-10.750]	0.55 [0.215-1.59]	<0.001
SAA (mg/L)	3.18 [1.61-11.2]	1.67 [0.96-2.82]	<0.001
IL-6 (pg/ml)	13.81 [4.31-41.33]	4.25 [1.19-18.17]	<0.001
TNF- α (pg/ml)	5.41 [2.75-10.92]	3.38 [2.37-4.76]	<0.001

Data presented as median [interquartile range] or percentage (%). P-values for Wilcoxon rank sum test for continuous variables and Pearson's chi-square test for categorical variables. HOMA-IR= homeostasis model assessment of insulin resistance. CAC= coronary artery calcium score.

Table 2
Correlation between serum free fatty acids and measures of inflammation, insulin resistance, coronary atherosclerosis in RA and control subjects

	RA		Control	
	Rho ^a	p-value	adjusted ^b p-value	Rho ^a p-value
Lipids				
Total cholesterol	0.142	0.068	0.006*	0.127 0.231 0.070
LDL cholesterol	0.099	0.205	0.033*	-0.029 0.787 0.532
HDL cholesterol	-0.014	0.858	0.600	0.161 0.128 0.226
Triglycerides	0.154	0.048*	0.005*	0.200 0.057 0.003*
Insulin resistance				
HOMA-IR	0.198	0.011*	0.013*	0.253 0.016* 0.016*
Inflammatory markers				
CRP	0.253	0.001*	0.010*	0.255 0.015* 0.218
SAA	0.185	0.017*	0.081	0.079 0.458 0.453
IL-6	0.056	0.481	0.755	-0.115 0.29 0.139
TNF α	0.084	0.288	0.166	0.111 0.296 0.645
ESR	0.090	0.249	0.358	- - -
Coronary artery disease				
FRS	0.215	0.005*	0.048*	0.281 0.007* 0.169
CAC	0.040	0.617	0.359	0.013 0.904 0.295
Other				
BMI	0.155	0.046*	0.281 ^c	0.2 0.006* 0.007* ^c
DAS28 Score	0.055	0.485	0.859	- - -

^a Spearman's correlation coefficient.

^b Derived from multivariable regression models that adjusted for age, sex, race and BMI.

^c Derived from multivariable regression models that adjusted for age, sex and race.

* p<0.05

HOMA-IR= Homeostasis Model Assessment of Insulin Resistance, CRP=C-Reactive Protein, SAA=Serum Amyloid A, IL-6=Interleukin 6, TNF α =Tumor Necrosis Factor- α , ESR=Erythrocyte Sedimentation Rate, FRS=Framingham Risk Score, CAC= Agatston coronary artery calcium score, BMI=Body Mass Index, DAS28=Disease Activity Score 28 Joint Count.

Table 3
Effect of medication use on serum FFAs in RA patients

Medication	FFAs median		p-value adjusted ^a	p-value
	Current use	No Current use		
Methotrexate	0.550 [0.375-0.725]	0.565 [0.387-0.81]	0.512	0.323
Hydroxychloroquine	0.500 [0.377-0.788]	0.570 [0.387-0.732]	0.759	0.974
Prednisone	0.57 [0.39-0.77]	0.54 [0.38-0.73]	0.719	0.369
Anti-TNF class	0.510 [0.37-0.748]	0.565 [0.398-0.748]	0.6	0.518
Statin class	0.58 [0.37-0.70]	0.56 [0.56-0.75]	0.708	0.4

Data presented as median [interquartile range].

^a adjusted for age, sex, race and BMI.