Leukocytes and complement in atherosclerosis

Alipour, A.

b. The effects of acute glucose loading on leukocyte activation in familial hyperlipidemic disorders

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ABSTRACT

Introduction: Chronic hyperlipidemia and hyperglycemia have been associated with leukocyte activation and atherosclerosis. The effect of acute glycemia on leukocyte activation *in vivo* has not been established yet. We compared leukocyte activation during an oral glucose tolerance test (OGTT) in patients with familial hypercholesterolemia (FH) and familial combined hyperlipidemia (FCH) compared to healthy controls.

Methods: Classical cardiovascular risk factors as well as leukocyte activation markers (CD11b and CD66b expression) were determined in the fasting state and during 2 hours post-load. The response during the OGTT was calculated as the incremental area under the curve (dAUC). Leukocyte activation markers were measured by flowcytometry using fluorescent labelled antibodies.

Results: The postprandial glucose response was significantly higher in FH and FCH compared to controls. The postprandial monocyte CD11b expression in controls showed a more pronounced decline (-12.94±2.08) than in FH (-5.42±1.18) and FCH (-6.29±2.28) (P=0.01 by ANOVA). The postprandial neutrophil CD11b response was different between the groups with a decrease in controls (-4.11±2.79), but unchanged in FH (1.67±1.33) and FCH (2.78±1.72) (ANOVA P=0.04). A similar trend was observed for neutrophil CD66b. Correction for the effects of lipid lowering drugs used in FH and FCH showed that there were no differences between users and non-users for fasting and postprandial leukocyte activation, suggesting that medication did not influence the postprandial responses. There was a consistent positive correlation between postprandial leukocyte activation markers and glucose excursions.

Conclusions: These data demonstrate that acute glycemia results in a suppression of monocyte and neutrophil activation in healthy subjects, in contrast to FH and FCH subjects who have a tendency for persistent activation. The differences may be explained by a more efficient postprandial glucose handling in controls than in FH and FCH.

INTRODUCTION

Atherosclerosis is considered to be a low-grade chronic inflammatory condition. In this situation, resident and recruited leukocytes and cytokines play crucial roles (1).
Both, hyperlipidemia and hyperglycemia are factors responsible for the inflammatory response in atherosclerosis and have been associated to endothelial and leukocyte activation (2-9). Several studies have shown that triglycerides (TG) and glucose can activate leukocytes and endothelial cells *in vivo* (3,4,6). However, glucose may not be a potent leukocyte activator in non-diabetic patients (4,10). Triglyceride-rich lipoproteins can activate leukocytes in the acute situation in subjects with and without atherosclerosis during postprandial lipemia, whereas acute hyperglycemia *in vitro* (15 minutes) does not induce leukocyte activation in healthy volunteers (4). *In vivo* studies from our group in young lean healthy men showed an increase of leukocytes during an oral glucose tolerance test (OGTT) (10), but this increase was much lower than the effect of fat in the acute phase.
Patients with familial hypercholesterolemia (FH) and familial combined hyperlipidemia (FCH) have higher levels of leukocyte activation and oxidative stress status than healthy controls (11-13). The increased pro-inflammatory state in these subjects has been linked to atherosclerosis (14,15). No data are available on postprandial leukocyte activation by acute hyperglycemia in patients with FH and FCH.
The aim of the present study was to evaluate the acute effect of glucose ingestion on leukocyte activation in patients with FH and FCH compared to healthy controls.

MATERIALS AND METHODS

Subjects

Subjects who visited the outpatient clinic of the department of Vascular Medicine of the Sint Franciscus Gasthuis and met the diagnostic criteria for FH and FCH were asked to participate. Also (untreated) subjects who were referred for cardiovascular risk screening and management were included if they met the criteria described below. Healthy volunteers were recruited by means of advertisement. FH was defined as having met the diagnostic criteria as outlined by the World Health Organization (16). FCH was defined as following: familial hyperlipidemia with a dominant inheritance pattern, elevated plasma apoB concentrations (>1.2 g/L) and elevated triglyceride (TG) levels (>1.7 mmol/L) at the time of diagnosis (17).
Exclusion criteria were: The presence of inflammatory disorders like rheumatoid arthritis, systemic lupus erythematosus and infections, plasma CRP above 10 mg/L, disorders of kidney, liver and thyroid function.

The Independent Ethics Committee of the Institutional Review Board of the St. Franciscus Gasthuis in Rotterdam and the regional independent medical ethical committee at the Maasstad Hospital in Rotterdam approved the study. The participants gave written informed consent.

Study design

During the first visit the cardiovascular history, anthropometric measures and the use of medication were recorded.
All participants visited the hospital after an overnight fast of at least 12 hours, without drinking alcohol on the day before and without taking their regular medication. Before the OGTT subjects rested for 30 minutes. The OGTT consisted of 75 g of oral anhydrous glucose. During each test, the participants remained in the hospital in a sitting position and were not allowed to drink or eat. Blood samples were obtained before the OGTT and after 1 and 2 hours postprandially from a peripheral vein of the forearm, and were kept on ice for further processing. The 2 hrs time point was chosen because the 2-hour blood glucose concentration after a glucose load is associated with adverse cardiovascular outcomes in type 2 diabetes and control patients (18,19). For leukocyte activation markers, blood samples were obtained in potassium EDTA (2 mg/mL).

Analytical methods

All clinical chemistry measurements were performed on the same day as the diagnostic coronary angiography. Basic parameters for renal and liver function as well as glucose, CRP, total cholesterol, HDL cholesterol and TG were determined using a Synchron LX analyzer (Beckman Coulter, Brea CA, USA) according to standard procedures in our laboratory for clinical chemistry. LDL cholesterol values were calculated using the Friedewald formula. Apolipoprotein (apo) AI and apoB were determined by rate nephelometry using IMMAGE with kits provided by Beckman (Beckman Coulter, Brea CA, USA). Blood cell counts were determined using the LH analyzer (Beckman Coulter, Miami FL, USA). The leukocyte differentiation was determined as a five-part differentiation on the same instruments.

Leukocyte activation markers

Blood samples for the measurement of leukocyte activation markers were collected in EDTA and were determined by flowcytometry on the same day. In order to differentiate leukocytes in lymphocytes, monocytes and neutrophils a CD45 (Immunotech Coulter, Marseille, France) versus SS gating strategy was used. Lymphocytes were defined as CD45 positive and low sideward scatter. Monocytes were defined as CD45 positive and intermediate sideward scatter. Neutrophils were defined as CD45 weak and high sideward scatter. The gates were set quite narrow for optimal differentiation of these cell populations rather than for completeness. For

tube 1 twenty µL blood from an EDTA-anti-coagulated blood sample was added to 2.5 µL of each CD66b FITC (Immunotech Coulter, Marseille, France), CD11b PE (Immunotech Coulter, Marseille, France) and CD45 ECD (Immunotech Coulter, Marseille, France). Cells were incubated for 15 minutes in the dark at room temperature. Erythrocytes were lysed by adding 300 µL of ice-cold isotonic erythrocyte lysing solution (NH4Cl 0.19M; KHCO3 0.01M; Na2EDTA•2H2O 0.12M, pH 7.2) for 15 minutes. A Coulter Epics XL-MCL flowcytometer with a 488nm Argon ion laser and EXPO 32 software was used for measurement and analysis. Cells were acquired during 2 minutes per sample. On average a total of 25.000 leukocytes per sample were measured. Fluorescence intensity of each cell was expressed as the mean fluorescence intensity (MFI), given in arbitrary units (AU). Additional experiments (data not shown) did not show significant differences between EDTA and heparin anti-coagulated blood for CD11b and CD66b expression. Furthermore we also did not find significant differences of CD11b and CD66b expression in a protocol in which we did not use ammoniumchloride for erythrocyte lysis (data now shown) .

Statistics

Data are given as mean±SEM in the text, in the Tables and in the Figures. The areas under the curve (AUC) for glucose, TG and the inflammatory markers were calculated by the trapezoidal rule using Graphpad Prism version 4.0 (LA, USA). Incremental integrated AUC's (dAUC) were calculated after correction for baseline values. Differences were tested by analysis of Variance (ANOVA) or Student's t-test where indicated. Fisher's Exact Test was used to evaluate dichotomous variables. Correlation analysis was carried out using Spearman correlation statistics. *P* values < 0.05 (2-tailed) were considered statistically significant.

RESULTS

Baseline characteristics (Table 1)

A total of 54 subjects divided into 3 groups (15 healthy volunteers, 22 FH and 17 FCH patients) participated in the study.

The baseline characteristics and cardiovascular risk factors are listed in Table 1. No differences were found for gender distribution, nor for smoking behavior between the groups. FH and FCH patients were older and had a higher systolic blood pressure and HbA1c than controls. FCH patients had a higher waist circumference and fasting glucose and TG levels when compared to controls and FH patients. HDL and apoAI levels were highest in FH patients. The other variables did not differ between the groups (Table 1).

The control subjects did not use any cardiovascular medication. Statin use in FCH and FH patients was 59.1 and 70.6% (P=0.34), and for ezetimibe 31.8 and 52.9% (P=0.16), respectively.

Table 1. General characteristics of controls and patients with FH and FCH.

	Controls (n=15)	FH (n=22)	FCH (n=17)	P-value
Gender (% women)	66.7	59.0	35.3	0.17
Smoking (% smokers)	6.7	9.0	17.6	0.23
Age (years)	49.47 (1.24)*	54.68 (1.30)	53.29 (1.43)	*0.03*
BMI (kg/m^2)	25.59 (1.24)	25.62 (0.79)	27.82 (0.75)	0.16
Waist circumference (m)	0.92 (0.04)	0.92 (0.02)	1.02 (0.02)†	*0.01*
BPsyst (mmHg)	116.9 (2.6)*	126.1 (1.9)	127.4 (3.5)	*0.02*
BPdiast (mmHg)	73.7 (1.7)	75.5 (1.5)	78.2 (1.4)	0.15
Glucose (mmol/L)	4.95 (0.11)	5.05 (0.11)	5.57 (0.10)†	*0.001*
HbA1c (%)	5.27 (0.08)*	5.62 (0.08)	5.61 (0.07)	*0.005*
TG (mmol/L)	0.96 (0.09)	1.13 (0.09)	2.70 (0.50)†	*<0.0001*
Total Cholesterol (mmol/L)	5.16 (0.17)	5.87 (0.34)	5.63 (0.33)	0.28
LDL-C (mmol/L)	3.29 (0.16)	3.63 (0.32)	3.11 (0.33)	0.44
HDL-C (mmol/L)	1.44 (0.11)	1.74 (0.11)‡	1.11 (0.06)†	*<0.0001*
ApoB (g/L)	0.95 (0.05)	1.13 (0.07)	1.17 (0.09)	0.13
ApoAI (g/L)	1.51 (0.08)	1.73 (0.08)‡	1.33 (0.05)	*0.001*
Platelet counts (10^9 cells/L)	245 (12)	270 (11)	246 (18)	0.30
Leukocyte counts (10^9 cells/L)	6.23 (0.38)	6.23 (0.33)	7.34 (0.53)	0.11
Neutrophil counts (10^9 cells/L)	3.53 (0.28)	3.46 (0.24)	4.20 (0.44)	0.21
Monocyte counts (10^9 cells/L)	0.51 (0.05)	0.51 (0.03)	0.58 (0.06)	0.52
Lymphocyte counts (10^9 cells/L)	1.97 (0.15)	2.06 (0.11)	2.36 (0.17)	0.17
C- reactive protein (mg/L)	1.73 (0.28)	1.82 (0.31)	2.76 (0.65)	0.21

Data are mean (SEM); BPsyst: systolic blood pressure. BPdiast: diastolic blood pressure. *: P<0.05 vs. FH and FCH patients. †: P<0.05 vs. Controls and FH patients; ‡: P<0.05 vs. controls and FCH patients.

None of the FH patients were on fibrates. Three patients in the FCH group used fibrates. FCH and FH patients not on lipid lowering drugs were new referrals to our department.

Baseline and postprandial leukocyte activation markers (Tables 2&3; Figures 1-3)

Table 2 shows the baseline leukocyte activation markers at T=0. No differences were found between controls, FH and FCH patients for the expression of monocyte and neutrophil CD11b and neutrophil CD66b.

Table 3 shows the changes in glucose, TG and inflammatory parameters in the three groups. Postprandial glucose changes were both significantly different between the groups with the lowest response in controls and the highest in FCH patients (Table 3, Figure 1).

There was a significant postprandial increase in neutrophil counts in controls and FH patients, whereas neutrophil counts in FCH patients remained unchanged (Table 3, Figure 2B). Total postprandial leukocyte (Figure 2A), monocyte (Figure 2C) and lymphocyte counts (Figure 2D), as well as platelet counts and CRP did not differ between the groups (Table 3).

Figure 3 shows the relative changes of postprandial leukocyte activation markers in the three groups. FH and FCH patients showed a lesser decrease in postprandial monocyte CD11b

Table 2. Leukocyte activation markers at baseline in controls and patients with FH and FCH.

	Controls (n=15)	FCH (n=17)	FH (n=22)	P-value
Monocyte CD11b (AU)	36.67 (1.95)	38.62 (2.35)	38.25 (1.66)	0.91
Neutrophil CD11b (AU)	29.09 (1.25)	33.35 (2.10)	30.35 (1.98)	0.46
Neutrophil CD66b (AU)	6.53 (0.52)	7.18 (0.38)	6.90 (0.72)	0.92

Data are mean (SEM).

Table 3. Effect of glucose during an OGTT, expressed as area under the curve (AUC) and the incremental area under the curve (dAUC) in controls and patients with FH and FCH.

	Controls (n=15)	FH (n=22)	FCH (n=17)	P-value
Glucose AUC (mmol.h/L)	9.48 (0.41)*	12.09 (0.44)	15.36 (0.85)†	*<0.0001*
Glucose dAUC (mmol.h/L)	-0.20 (0.25)*	1.99 (0.43)	3.96 (0.72)†	*<0.0001*
TG AUC (mmol.h/L)	1.83 (0.16)*	2.22 (0.20)	5.68 (0.99)†	*0.0001*
TG dAUC (mmol.h/L)	-0.09 (0.04)	-0.03 (0.04)	0.34 (0.48)	0.47
Platelets AUC (*10^9.h/L)	482.57 (24.32)	535.9 (22.8)	481.76 (33.37)	0.23
Platelets dAUC (*10^9.h/L)	-6.63 (4.21)	-5.16 (3.74)	-14.00 (4.42)	0.33
Leukocytes AUC (*10^9.h/L)	12.23 (0.74)	12.01 (0.59)	13.69 (0.87)	0.22
Leukocytes dAUC (*10^9.h/L)	-0.23 (0.17)	-0.45 (0.17)	-0.75 (0.15)	0.14
Neutrophils AUC (*10^9.h/L)	7.54 (0.58)	7.22 (0.49)	8.37 (0.80)	0.40
Neutrophils dAUC (*10^9.h/L)	0.47 (0.14)	0.30 (0.09)	-0.04 (0.14)†	*0.02*
Monocytes AUC (*10^9.h/L)	0.94 (0.10)	0.95 (0.08)	0.94 (0.13)	1.00
Monocytes dAUC (*10^9.h/L)	-0.07 (0.07)	-0.01 (0.09)	-0.02 (0.13)	0.89
Lymphocytes AUC (*10^9.h/L)	3.42 (0.25)	3.56 (0.16)	3.95 (0.29)	0.27
Lymphocytes dAUC (*10^9.h/L)	-0.53 (0.10)	-0.56 (0.11)	-0.70 (0.11)	0.53
Monocyte CD11b-AUC (AU.h/L)	61.73 (2.54)	71.08 (3.51)	71.13 (3.41)	0.10
Monocyte CD11b-dAUC (AU.h/L)	-12.94 (2.08)*	-5.42 (1.18)	-6.29 (2.28)	*0.01*
Neutrophil CD11b-AUC (AU.h/L)	56.23 (3.17)	62.38 (3.92)	69.47 (4.14)	0.09
Neutrophil CD11b-dAUC (AU.h/L)	-4.11 (2.79)*	1.67 (1.33)	2.78 (1.72)	*0.04*
Neutrophil CD66b-AUC (AU.h/L)	13.15 (1.20)	13.44 (1.28)	14.54 (0.85)	0.70
Neutrophil CD66b-dAUC (AU.h/L)	-0.62 (0.32)	-0.37 (0.26)	0.08 (0.31)	0.27
CRP AUC (mg.h/L)	3.33 (0.48)	3.57 (0.59)	11.78 (6.43)	0.18
CRP dAUC (mg.h/L)	-0.13 (0.19)	-0.07 (0.11)	0.11 (0.22)	0.93

Data are mean (SEM); *: P<0.05 vs. FH and FCH patients; †: P<0.05 vs. Controls and FH patients.

expression than the controls, resulting in a significantly lower monocyte CD11b-dAUC in controls than in FH and FCH patients (Table 3, Figure 3A). Neutrophil CD11b showed an early suppression at 1 hour, only in controls (Figure 3B). Such a pattern was also observed for the postprandial neutrophil CD66b expression, but it did not reach statistical significance (Table 3, Figure 3C).

In order to analyze the effects of lipid lowering drugs, the data of FH and FCH subjects were combined. This was done due to the similarities in postprandial leukocyte activation response in these groups. The total group was subdivided into statin users (n=25) and non-users (n=14), ezetimibe users (n=16) and non-users (n=23) and fibrate users (n=3) and non-users (n=36). There were no differences for leukocyte activation markers at fasting state nor for postprandial values between these groups.

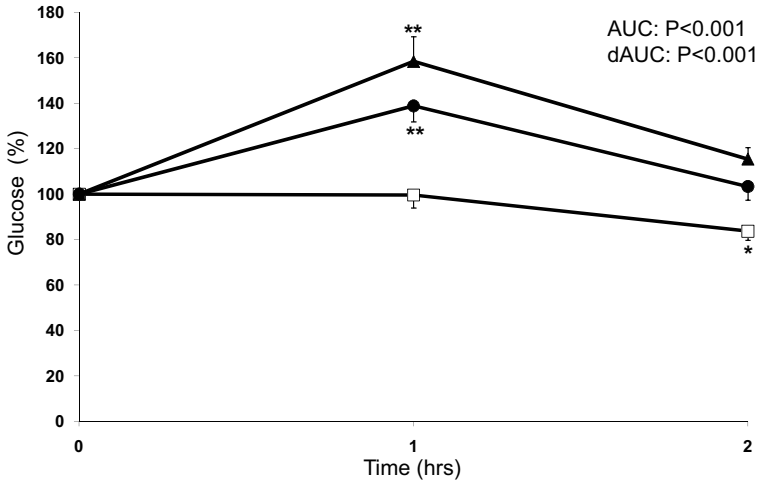

Figure 1. Mean±SEM relative changes of glucose after ingestion of oral glucose in healthy controls (open square) and patients with FH (closed circles) and FCH (closed triangle). *: P=0.001 vs. T=0, **: P<0.001 vs. T=0.

Determinants of leukocyte activation markers

There were no significant correlations between fasting monocyte and neutrophil CD11b and neutrophil CD66b expression and the classical cardiovascular risk factors. Monocyte CD11b-dAUC correlated positively with glucose-dAUC (R=0.44, P=0.001), with a trend for plasma apoB (R=0.25, P=0.07). There was a positive correlation for neutrophil CD11b-dAUC with glucose-dAUC (R=0.28, P=0.04) and plasma apoB (R=0.42, P=0.002). Neutrophil CD66b-dAUC correlated positively with plasma apoB (R=0.32, P=0.02), with a trend for glucose-dAUC (R=0.24, P=0.08).

DISCUSSION

To the best of our knowledge, this is the first study describing acute changes in leukocyte activation by glucose in patients with FH and FCH compared to healthy controls. Triglyceride-rich lipoproteins are potent activators of leukocytes (2-5), but the effect of glucose on leukocytes is less consistent (4,6,7). Previously, Sampson et al showed a 4% increase of CD11b in controls following an OGTT (6). In contrast, it has been shown that hyperglycemia reduces neutrophil degranulation in septic patients (20). Therefore, the effects of glucose on leukocyte activation may depend on the clinical situation and the type of patients studied.

CD11b and CD66b are distinguished markers of atherosclerosis. The expression of these markers has been linked directly to atherosclerosis (21-23). CD11b (also termed MAC-1 or CR3) is involved in early adhesion of leukocytes to the endothelium and CD66b (also termed

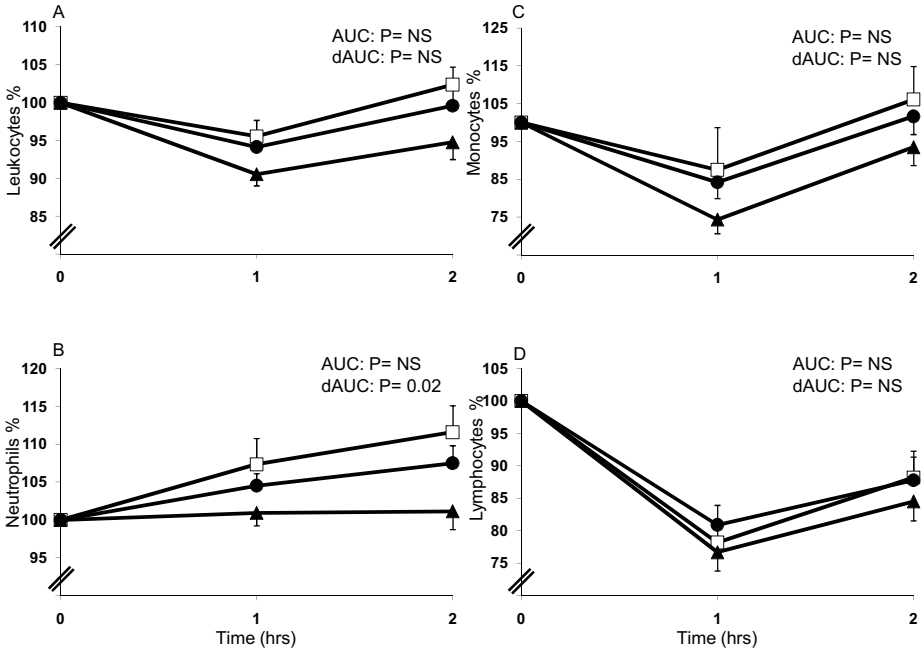

Figure 2. Mean±SEM relative changes of total leukocyte (A), neutrophil (B), monocyte (C) and lymphocyte (D) counts during a 2 hours OGTT in healthy controls (open square) and patients with FH (closed circles) and FCH (closed triangle).

CEACAM8) is a marker of degranulation (24,25). Fasting leukocyte activation markers did not differentiate between the groups. The differences were revealed in the postprandial situation. The normal response to *acute* glycemia seems to be a suppression of the expression of CD11b and CD66b as illustrated by our data in healthy subjects. FH and FCH patients showed an unaltered expression of CD11b and CD66b on neutrophils and a blunted suppression of monocyte CD11b compared to the controls. These results are in line with a pro-inflammatory situation in these patients as described by others (11-13). It is of interest that our results were obtained in treated subjects with LDL, apoB and glucose levels within normal ranges and similar to controls. Our sub-analyses based on the use of medication showed no differences on leukocyte activation. We can not draw definitive conclusions on the effects of medication at this point because of the small number of patients included without medication. Previous studies have shown that statins can influence fasting leukocyte activation (11,26,27). Ezetimibe inhibits monocyte migration in a rabbit model (28), but no data are available on its effects on the expression of CD11b and CD66b. Moreover, it has been shown that fibrates are able to suppress the pro-inflammatory cytokine release from monocytes (29), but its effects on CD11b and CD66b are unknown.

The best association between postprandial leukocyte activation and potential determinants was plasma apoB, but also postprandial glucose response. It is of interest to note that both

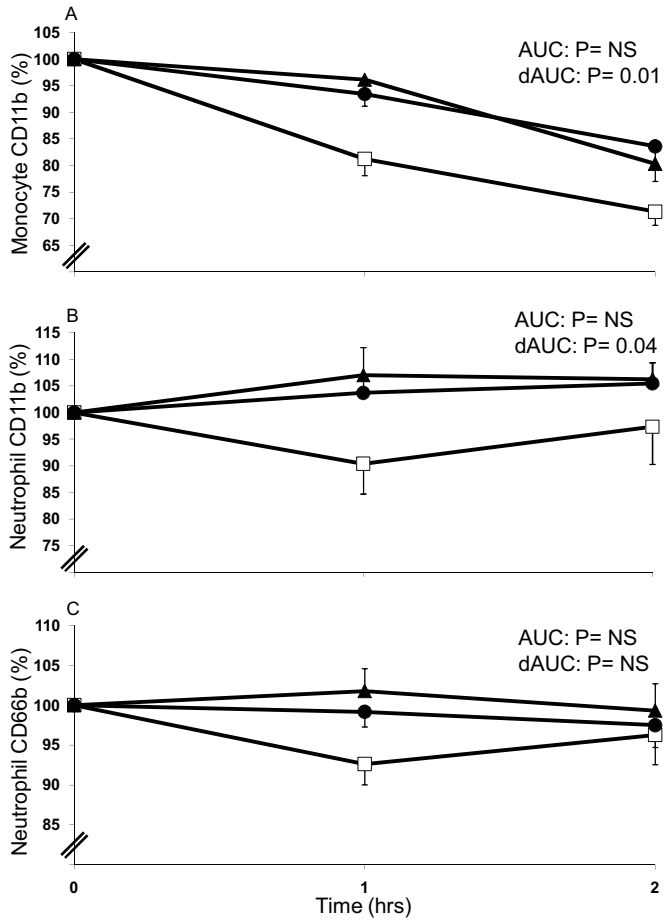

Figure 3. Mean±SEM relative expression of monocyte CD11b (A), neutrophil CD11b (B) and CD66b (C) during a 2 hours OGTT in healthy controls (open square) and patients with FH (closed circles) and FCH (closed triangle).

FCH and FH showed a significantly higher postprandial glucose response compared to controls. Since FCH patients are known to be insulin resistant (30-32), this was not surprising. Only limited data are available in FH patients suggesting that this is not an insulin resistant state based on euglycemic hyperinsulinemic clamps (33). However, our OGTT data are very similar to those presented in that study and point at a less efficient glucose metabolism in FH. It remains to be shown whether improved glucose handling in FH and FCH will lead to less postprandial leukocyte activation.

We did not find a difference for CRP between the groups. Statins have been shown to decrease CRP levels (34). Thus, the use of statins could have been a confounding factor in that respect. In addition, we did not use a high sensitive CRP assay and this may explain the lack of differences between the groups.

In conclusion, the present study shows that fasting measures of leukocyte activation markers do not differentiate between patients with FH and FCH from controls. Acute glycemia results in suppressed monocyte and neutrophil activation in healthy controls, whereas hyperlipidemic patients, e.g. patients with FH and FCH, under standard therapy had a persistent activation of leukocytes. This difference between the hyperlipidemic patients and controls seems to be related to a more efficient glucose metabolism in controls. It remains to be shown whether the response in hyperlipidemic patients can be restored by improving insulin sensitivity.

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Disclosures
None declared.

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