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Toll Like Receptor 2 and 4 stimulation elicits an enhanced inflammatory response in human obese patients with atherosclerosis.

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Abstract

Objective. The innate immune response elicited by activation of toll like receptors (TLR) plays an important role in the pathogenesis of atherosclerosis. We hypothesized that cardiovascular risk factors are associated with the activation status of the innate immune system. We therefore assessed the responsiveness of TLR's on circulating cells in 2 groups of patients with established atherosclerosis and related this to the presence of cardiovascular risk factors.

Methods. TNF-alpha release induced by TLR 2 and 4 activation was measured in patients with established coronary (PCI study n=78) or carotid artery disease (CEA study n=104), by stimulating whole blood samples with Lipopolysaccharide (TLR4) and Pam3Cys-Ser-(Lys)4 (TLR2). As an early activation marker, CD11b expression was measured by flow-cytometry on CD14 positive cells.

Results. Obesity was the *only* risk factor that correlated with TLR response. In both studies, obese patients had significantly higher TNF- alpha levels after stimulation of TLR2, compared with non-obese patients (median [IQR]pg/ml): coronary artery disease 16.9 [7.7 – 49.4] versus 7.5 [1.5 – 19.2] (p = 0.008) and carotid artery disease 14.6 [8.1 – 28.4] versus 9.5 [6.1 – 15.7] (p = 0.015). Similar results were obtained following TLR4 stimulation. The enhanced inflammatory state in obese patients, was also confirmed by an significant increased expression of the activation marker CD11b on circulating monocytes.

Conclusion. Obesity is associated with an enhanced TLR response in patients suffering from established atherosclerotic disease.

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Introduction

Atherosclerosis is considered as a generalized chronic inflammatory disorder of the vascular tree [1, 2]. Patients suffering from chronic inflammatory diseases, like systemic lupus erythematosus or rheumatoid arthritis have an increased risk for developing atherosclerosis [3, 4]. A potential clue for the underlying mechanisms of this increased prevalence, might be the chronic inflammatory state to which patients are subjected to. This enhanced systemic inflammatory state drives the successive activation of endothelial cells, leucocyte adherence and migration through the arterial wall leading to the production of pro-inflammatory cytokines. The relevance of smoking, diabetes, hypercholesterolemia and hypertension in the development of atherosclerosis is evident [5]. A possible explanation for the increased risk of atherosclerosis in the presence of these traditional risk factors, is the enhanced activation of the immune system.

Obesity is a worldwide increasing problem, especially in Western societies [6-8]. Obesity is associated with an increased risk of acquiring traditional cardiovascular risk factors, potentiating the development of atherosclerosis and concomitant cardiovascular complications [9-11]. Obesity is also directly related to the development and progression of atherosclerosis [12-15]. Several articles provided evidence that adipose tissue is not just an energy storage depot but also a source of cytokine producing inflammatory cells [16-20].

Toll like receptors, which are part of the innate immune system, are pattern recognizing receptors forming the first line of defense, quickly reacting on evolutionary conserved pathogens [21]. But also endogenous ligands like extra domain A, heat shock proteins, fibronectin and free fatty acids are able to activate these receptors resulting into a pro-inflammatory cytokine response. TLR's are found in atherosclerotic tissue but also have shown to play a role in lipid uptake in macrophages and are expressed on circulating monocytes [22, 23]. We and others have shown the importance of these receptors in the initiation and progression of atherosclerosis [24-27]. Responsiveness upon ligation of these receptors can be measured in whole blood, *ex vivo*. Previously, we showed that responsiveness altered with different severities of coronary artery stenosis [28]. Others showed that this response was elevated in patients having unstable angina pectoris or acute myocardial infarction, compared with patients with stable angina, [29, 30].

Considering the central role the Toll Like Receptor in atherosclerosis development, we hypothesized that cardiovascular risk factors are associated with an altered activation status of the innate immune system, reflected by differences in TLR responsiveness. We assessed the TLR response in patients with established atherosclerosis in two different territories: atherosclerosis of the coronary (percutaneous coronary intervention, PCI study) and of the carotid artery (carotid endarterectomy, CEA study) and related this to the presence of cardiovascular risk factors. In addition, we measured expression of the early activation marker CD11b on circulating monocytes.

We demonstrate that obesity is associated with an altered TLR response in patients with established atherosclerosis of the coronary or carotid artery.

Material and Methods.

Patients

Two cohorts of patients were analyzed for this study. Both studies were approved by the local medical committee board and informed consents were obtained.

The first cohort consisted of 100 subsequent patients scheduled for Percutaneous Coronary Intervention (PCI study) at the University Medical Centre Utrecht.

The second group consisted of 111 consecutive patients scheduled for carotid artery endarterectomy (CEA study) at the University Medical Centre Utrecht and at the St Antonius Hospital, Nieuwegein.

This last patient group is part of the Athero-express Biobank study [31].

Clinical data were obtained from patients medical file records and from pre-procedural filled-in questionnaires. Risk factors were scored as follows: Hypertension: the use of one or more antihypertensive drugs (> 3 months before intervention); Hypercholesterolemia: the use of statins for at least 3 months before intervention; Diabetes: the use of anti-diabetic medication (> 3 months before intervention); Positive family history of heart disease: > 1 family member with a cardiac event (MI, AP or CABG) < 60 years. Obese patients were classified as having a body mass index (BMI) > 25.0 and non-obese patients as having a BMI ≤ 25.0. Smoking status was derived from the preoperative intake questionnaire of the department of anesthesiology.

Blood sampling

Percutaneous Coronary Intervention (PCI) study. Whole blood samples were drawn in lithium-heparin (LH) and ethylenediaminetetraacetic acid (EDTA) anticoagulated tubes before PCI. To prevent premature leucocyte activation all tubes were kept on ice until further processing. The effect of short term preservation on ice on cytokine production, was evaluated before initiation of this study. This short term preservation did not influence the experiments. 100 µl of blood was stimulated with 100 µl of the synthetic TLR2 ligand Pam3Cys-SK4 (P3C) (Novabiochem, Cambridge, MA, USA) at 500 ng/ml and 100 µl of TLR4 ligand LPS (derived of E. coli serotype O55:B5) (Sigma, St Louise, MO, USA) at 10 ng/ml. Samples were incubated overnight (o/n) at 37° C and 5% CO₂. After o/n incubation, samples were centrifuged at 1000 rpm for 5 minutes and supernatants were collected and stored at -20°C until further processing. As a surrogate of TLR2 and TLR4 response, TNF-alpha was measured in the supernatants using an enzyme linked immunosorbent assay (ELISA) according to the manufacturers protocol (Pelikine-compact, Sanquin, Amsterdam, the Netherlands). TNF-alpha levels were corrected for white blood cell count, which was measured using a hematology analyzer (Celldyn 1700, Abbott Diagnostics, IL, USA).

In EDTA anti-coagulated blood samples, TLR2, TLR4 and CD11b expressions on CD14 positive monocytes were determined by flow cytometry (Cytomics FC500, Beckman Coulter, CA, USA). Whole blood samples were stained for CD14 (PE-Cy5) combined with either TLR2 (FITC) and TLR4 (PE) or CD11b (FITC) (all Serotec, Oxford UK). TLR2, TLR4 and CD11b expression levels are referred to as mean fluorescence intensity (MFI) on CD14 positive monocytes. C reactive protein levels in serum were determined using a Nefelometer (Dade Behring, now Siemens Healthcare Diagnostics, Germany), performed by the clinical chemistry laboratory of the UMC Utrecht hospital.

Carotid End-Arterectomy (CEA) study. Whole blood samples were drawn before incision, immediately after anesthesia. In a sub study the effect of anesthetics was investigated on TLR activation. The anesthetics did not influence the TNF-alpha levels (data not shown). Samples were stored in heparinized tubes and immediately processed at the laboratory. Blood samples were stimulated by using the same protocol as used in the PCI study, but instead of overnight incubation, samples were stimulated for 2 hours. As a surrogate of TLR2 and TLR4 response, TNF-alpha, IL6 and IL8 were measured in the supernatants using a multiplex immunoassay system according to the manufacturers protocol, (BioPlex 200, BioRad). TNF-alpha, IL6 and IL8 levels were corrected for white blood cell count.

In heparin anti-coagulated blood samples, TLR2, TLR4, CD11b expression on CD14 positive monocytes were examined by flow cytometry (Cytomics FC500, Beckman Coulter, CA, USA). Whole blood samples were stained for CD14 (CD14-RPE-Cy5) combined with TLR2 (FITC) and TLR4 (PE) (Serotec, Oxford, UK) and CD11b (CD11b-PE-Cy7) (eBiosciences, San Diego, USA). TLR2, TLR4 and CD11b expression levels are referred to as MFI on CD14 positive monocytes. CD 11b expression on CD14 + cells was also measured after stimulation. 100 μ l of heparinized whole blood was stimulated with 100 μ l of: 1 PBS (negative control), 2 Pam3Cys (500ng/ml) (TLR2 stimulation) 3 LPS (100ng/ml) (TLR4 stimulation) and 4 Phorbol 12-myristate 13-acetate (PMA) (aspecific stimulation). Stimulated blood samples were incubated for 30 minutes at 37° C and 5% CO₂.

Exclusion

In the PCI study, 22 patients were excluded for analysis: 9 patients suffering from acute myocardial infarction within 24 hours prior to PCI, 12 patients suffered from chronic inflammatory disorders receiving corticosteroid drugs and finally 1 patient was excluded because information about traditional risk factors was lacking.

In the CEA study, 7 patients were excluded for analysis: 4 patients with chronic inflammatory disorder receiving corticosteroid drugs preoperatively and 3 patients, because white blood cell counts were not performed.

Statistics

TNF-alpha levels, mean fluorescence levels and CRP levels were not normally distributed. After ln transformation, the data was normally distributed. Differences between two independent variables were calculated with the independent sample T- test. Differences between 3 or more independent variables were calculated by using the one-way ANOVA test. Differences between 2 dichotomous values were calculated with the Chi-square test. Differences between 2 continuous variables were calculated by using the Spearman test. A p value < 0.05 was considered as statistically significant. Multivariate correction was performed by using a linear regression model. Gender, age, hypertension, hypercholesterolemia, diabetes, smoking status, positive family history for cardiovascular disease, hsCRP levels and BMI < 25 or BMI > 25 were analyzed with a linear regression model. In the CEA study, hsCRP levels were not available and positive family history for cardiovascular disease was left out the model because missing data of 18 patients. All statistical analysis were performed with PASW statistics version 17 (SPSS inc, Chigago, IL, USA)

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Results

22 patients in the PCI study and 7 patients in the CEA study were excluded from analysis (for details, see the methods section). 78 (PCI study) and 104 patients (CEA study) were used for analysis. Clinical characteristics are depicted at table 1 and S1.

In both studies, TNF-alpha was used as a specific marker for TLR response. Several authors have demonstrated the TLR specificity of this cytokine[32, 33]. Stimulation of whole blood samples with PBS did not result into detectable TNF-alpha levels and in addition stimulation with specific TLR2 and TLR4 agonists Pam3Cys and LPS resulted in a dose-dependent TNF-alpha release (data not shown).

In both studies, obese patients showed significantly higher TNF-alpha levels after stimulation of Toll Like Receptor 2, compared with non-obese patients: (median [IQR] pg/ml, adjusted p-value) 16.9 [7.7 – 49.4] versus 7.5 [1.5 – 19.2] and 14.6 [8.1 – 28.4] versus 9.5 [6.1 – 15.7] ($p=0.008$ and $p=0.015$ for the PCI and CEA study, respectively) (fig 1). TNF-alpha levels in obese patients were also significantly higher compared with non-obese patients following TLR4 stimulation: 257.1 [129.0 – 368.7] versus 180.9 [91.2 – 236.6] (PCI study) $p=0.014$ and not significantly enhanced in the CEA study: 209.4 [118.9 – 340.2] versus 154.2 [105.4 – 260.9] ($p=0.396$). (fig 1). In the PCI study, we observed a trend towards lower levels of TNF-alpha following TLR2 and TLR4 stimulation for patients who were active smokers, compared with patients who do not smoke. However, this trend could not be observed in the CEA study (table 2). When taking other risk factors into account, we observed no differences in responsiveness between patients with or without these cardiovascular risk factors (table 2).

In the CEA study we also quantified the levels of IL6 and IL8 following TLR stimulation. Obese patients had compared with non-obese patients also increased levels of IL6 following TLR 2 and TLR 4 stimulation; (median [IQR] pg/ml, adjusted p-value) 7 [3 – 30] vs 4 [2 – 12] $p=0.045$ and 123 [47 – 240] vs 56 [31 – 133] $p=0.054$ respectively. IL8 levels were elevated in obese patients following TLR 2 stimulation, but not following TLR 4 stimulation. 68 [35 – 147] vs 37 [23 – 63] $p=0.042$ and 560 [306 – 857] vs 475 [226 – 730] $p=0.729$ respectively. (figure 4).

As a marker for early activation, CD11b expression was measured at baseline in a consecutive subset of patients in both studies. In the PCI and CEA study, the flow cytometry measurements were included in the study protocol at a later time point. In both studies, obese patients revealed increased CD11b expression on CD14 positive monocytes, compared with non-obese patients, and these differences were significant in the PCI study (fig 2). In the CEA study we also measured CD11b expression after TLR 2 and 4 stimulation and again, obese patients showed increased expression of the activation marker CD11b (fig 2). After correction for risk factors the differences observed between obese and non-obese patients remained significant ($p=0.009$ and $p=0.012$ after stimulation with LPS and Pam3Cys resp). Differences between groups were not observed after stimulation with an aspecific stimulus, Phorbol 12-myristate 13-acetate (PMA) (fig 2). We did not observe differences in CD11b expression between patients with other established risk factors (table S2). In the PCI study we measured high sensitive C-reactive protein (hsCRP). Obese patients demonstrated higher levels of hsCRP (fig 3). Finally, TLR2 and 4 expressions on circulating monocytes were quantified in both studies. In both studies we did not observe differences in TLR 2 and 4 expression between obese and non-obese patients: median [IQR] PCI study: TLR2 expression 3.0 [2.4 – 3.8] and 3.3 [2.8 – 4.0] and TLR4 expression 2.4 [2.1 – 3.6] and 2.8 [2.5 – 3.5] for non-obese and obese patients respectively. CEA study: TLR2 expression 1.5 [0.9 – 2.5] and 1.8 [1.0 – 4.3] and TLR4 expression 0.3 [0.2 – 0.5] and 0.5 [0.3 – 0.7] for non-obese and obese patients respectively (table S3).

Discussion

Toll like receptor activation results in an innate immune response and plays an important role in the pathogenesis of atherosclerosis. An association between cardiovascular risk factors and the activation status of the innate immune system, which is reflected by an altered TLR responsiveness, can be suggested. We demonstrate in two cohorts of patients with established atherosclerosis, that obesity is a cardiovascular risk factor related with an increased TNF-alpha production by white blood cells following TLR activation.

Obesity and inflammation

While obesity was primarily seen as a lipid storage disease, it is nowadays recognized that obesity should be considered as a chronic inflammatory disorder [16-18]. Several studies demonstrated that obese patients have increased levels of circulating biomarkers reflecting systemic inflammation [19, 34]. High sensitive CRP is one of the most commonly studied biomarkers and is found to be elevated in obese patients [34]. Our data also confirmed that obese patients, have increased levels of hsCRP (fig 3). Another activation marker of the immune cells is CD11b. CD11b forms together with CD18 the macrophage-1 antigen (Mac-1 or complement receptor 3) and is regarded as a key integrin in mononuclear cell adhesion towards activated endothelium [35]. In both studies obesity was associated with increased CD11b expression on CD14⁺ monocytes (fig 3). In the CEA study, CD11b expression was also measured after TLR stimulation, which resulted in significant differences between obese and non-obese patients. Stimulation with the aspecific stimulus PMA (positive control) did not show any differences between obese and non-obese patients.

Zhang et al. showed that physiological levels of Non Esterified free Fatty Acids (NEFA), which are elevated in obese patients, are able to up regulate CD11b expression on human monocytes and increase monocyte adherence on an adhesion assay [36]. CD11b is an important adhesion molecule, necessary for firm adhesion of the circulating monocyte to the activated endothelium. Enhanced expression of this integrin therefore might facilitate monocyte entry in atherosclerotic lesions. This could be one possible mechanism linking obesity with atherosclerosis.

Obesity and TLR response

In this study, obesity was the only risk factor that was associated with an increased TLR response. Obese patients have elevated levels of apolipoprotein CIII (apoCIII) and free fatty acids (FFA's) which are able to activate TLR 2 and 4 respectively [37, 38]. TLR's are mainly expressed on leucocytes, but also adipocytes and macrophages in adipose tissue express these receptors. Release of apoCIII or FFA's, activate TLR 2 and 4 leading to a pro-inflammatory cytokine production. This might be an explanation of the increased levels of circulating biomarkers reflecting systemic inflammation in obese patients. Secondly, monocytes activated by these elevated levels of apoCIII or FFA's, will express more CD11b, and might adhere more easily to the activated endothelium. Because of their already activated state, upon stimulation, these monocytes will produce elevated levels of pro-inflammatory cytokines resulting in more severe atherosclerosis development.

Obese patients are also at increased risk for developing insulin resistance. In a recent review, Konner et al. summarized the abundant evidence that links TLR activation with insulin resistance [39]. An important role for saturated FFA's causing insulin resistance in a TLR dependent manner was clearly highlighted. Saturated FFA's are able to activate inhibitor of nuclear factor (NF)-kB kinase (IKK) and c-Jun N-terminal kinase (JNK) in a TLR dependent manner. Activation of these important kinases not only results into transcription of pro-inflammatory cytokines (via NF-kB and AP-1) but also phosphorylates insulin receptor substrate (IRS) thereby causing insulin resistance. In a large genome wide association study, Weyrich et al. demonstrated an association between TLR4^{D299G/T399I} polymorphism and increased body fat and insulin resistance in 2 different cohorts, supporting the role of TLR4 as a molecular link between obesity and insulin resistance [40].

The enhanced responsiveness we observed in obese patients could not be explained by an elevated expression of TLR's on circulating monocytes. Therefore, other factors influencing TLR responsiveness might explain the increased responsiveness observed in obese patients. Schwarz et al. demonstrated that saturated free fatty acids, which are elevated in obese patients, are able to augment TLR induced cytokine production in a TLR independent manner [41]. They showed that the saturated fatty acids lauric acid, palmitic acid or stearic acid are able to augment TLR2 and TLR4 response by stimulation of Protein Kinase C. Another possible explanation could be the effect of the anti-inflammatory adipokine adiponectin, of which levels are decreased in obese patients [42]. Yamaguchi et al. demonstrated that adiponectin is able to inhibit TLR 2,4 and 9 induced NF-kB activation in murine macrophages[43]. In addition, another study showed decreased IL6 and TNF-alpha production of LPS stimulated porcine macrophages pretreated with adiponectin [44]. Although the exact mechanism is still unknown, the decreased levels of adiponectin in obese patients might explain the observed increased responsiveness in obese patients.

Smoking and TLR response

In the PCI study, we observed a trend towards lower levels of TNF alpha following TLR stimulation in patients who are active smokers (table 2). When taking into account the time patients had stopped smoking there was a trend towards higher TNF alpha levels for patients who had stopped > 6 months or had never smoked (table 2). This trend could not be observed in the CEA study. Chen et al showed decreased TLR 2 and 4 responsiveness in alveolar macrophages isolated from active smokers compared with healthy controls, but failed to demonstrate decreased responsiveness of smokers PBMC's, following 6 hours of stimulation [45]. We only observed the decreased TLR response in the PCI study, following a longer period of stimulation (overnight incubation). This might explain the differences we observed between our 2 studies and the study performed by Chen.

The strength of our study is that we demonstrated increased responsiveness in obese patients in two independent cohorts with established atherosclerosis of two different vascular territories. Another strength which is also a limitation of this study, is the different stimulation times used in both studies. It shows that differences can be observed between obese and non-obese patients despite changes in stimulation duration. On the other hand, we cannot state that we reproduced the results using an identical study design. Secondly, flowcytometry measurements were not performed after TLR stimulation in the PCI study. Finally, our study is descriptive and a control group of obese patients without cardiovascular disease is lacking. We can only speculate upon possible explanations for the increased responsiveness observed in obese patients. It would be of great interest to further explore this observation to see if healthy obese patients, without clearly established atherosclerosis, have also an increased TLR responsiveness. In addition, it would be interesting to see whether more specific markers of activation, like CD11c, are also elevated in obese patients.

Conclusion

Obese patients with established atherosclerosis show an increased whole blood TLR response and increased expression of the activation marker CD11b.

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Author Contribution

Dik Versteeg performed the PCI study, Vincent Scholtes performed the CEA study. Arjan Schoneveld provided technical supervision and set up the multiplex immunoassay system. Analyses were performed by Vincent Scholtes with help from Karlijn van Keulen and Imo Hofer. Pieter Stella and Pieter Doevendans are intervention cardiologist and together with Jean Paul de Vries and Frans Moll (vascular surgeons) made it possible to obtain blood prior to intervention. Gerard Pasterkamp designed the hypothesis of the study and supervised the entire study. Dominique de Kleijn, Jean-Paul de Vries and Gerard Pasterkamp revised the first versions of the manuscript. All of the authors had full access to the data and take responsibility for its integrity and the accuracy of the analysis. All authors have read and agree to the manuscript as written.

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References

1. Ross, R. (1999). Atherosclerosis--an inflammatory disease. *N. Engl. J. Med.* **340**, 115-126.
2. Stary, H.C., Chandler, A.B., Glagov, S., Guyton, J.R., Insull, W., Jr., Rosenfeld, M.E., Schaffer, S.A., Schwartz, C.J., Wagner, W.D., and Wissler, R.W. (1994). A definition of initial, fatty streak, and intermediate lesions of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation.* **89**, 2462-2478.
3. del Rincon, I.D., Williams, K., Stern, M.P., Freeman, G.L., and Escalante, A. (2001). High incidence of cardiovascular events in a rheumatoid arthritis cohort not explained by traditional cardiac risk factors. *Arthritis Rheum.* **44**, 2737-2745.
4. Kao, A.H., Sabatine, J.M., and Manzi, S. (2003). Update on vascular disease in systemic lupus erythematosus. *Curr. Opin. Rheumatol.* **15**, 519-527.
5. Wilson, P.W. (1994). Established risk factors and coronary artery disease: the Framingham Study. *Am J Hypertens.* **7**, 7S-12S.
6. WHO. (2002). Reducing risks, Promoting healthy life. (World Health Organisation, Geneva, 2002).
7. Flegal, K.M., Carroll, M.D., Ogden, C.L., and Curtin, L.R. (2010). Prevalence and trends in obesity among US adults, 1999-2008. *JAMA.* **303**, 235-241.
8. Lloyd-Jones, D., Adams, R., Carnethon, M., De Simone, G., Ferguson, T.B., Flegal, K., Ford, E., Furie, K., Go, A., Greenlund, K., Haase, N., Hailpern, S., Ho, M., Howard, V., Kissela, B., Kittner, S., Lackland, D., Lisabeth, L., Marelli, A., McDermott, M., Meigs, J., Mozaffarian, D., Nichol, G., O'Donnell, C., Roger, V., Rosamond, W., Sacco, R., Sorlie, P., Stafford, R., Steinberger, J., Thom, T., Wasserthiel-Smoller, S., Wong, N., Wylie-Rosett, J., and Hong, Y. (2009). Heart disease and stroke statistics--2009 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation.* **119**, 480-486.
9. Flegal, K.M., Graubard, B.I., Williamson, D.F., and Gail, M.H. (2007). Cause-specific excess deaths associated with underweight, overweight, and obesity. *JAMA.* **298**, 2028-2037.
10. Kenchaiah, S., Evans, J.C., Levy, D., Wilson, P.W., Benjamin, E.J., Larson, M.G., Kannel, W.B., and Vasan, R.S. (2002). Obesity and the risk of heart failure. *N. Engl. J. Med.* **347**, 305-313.
11. Yan, L.L., Daviglius, M.L., Liu, K., Stamler, J., Wang, R., Pirzada, A., Garside, D.B., Dyer, A.R., Van Horn, L., Liao, Y., Fries, J.F., and Greenland, P. (2006). Midlife body mass index and hospitalization and mortality in older age. *JAMA.* **295**, 190-198.
12. Cassidy, A.E., Bielak, L.F., Zhou, Y., Sheedy, P.F., 2nd, Turner, S.T., Breen, J.F., Araoz, P.A., Kullo, I.J., Lin, X., and Peyser, P.A. (2005). Progression of subclinical coronary atherosclerosis: does obesity make a difference? *Circulation.* **111**, 1877-1882.
13. Fantuzzi, G., and Mazzone, T. (2007). Adipose tissue and atherosclerosis: exploring the connection. *Arterioscler. Thromb. Vasc. Biol.* **27**, 996-1003.
14. McGill, H.C., Jr., McMahan, C.A., Herderick, E.E., Zieske, A.W., Malcom, G.T., Tracy, R.E., and Strong, J.P. (2002). Obesity accelerates the progression of coronary atherosclerosis in young men. *Circulation.* **105**, 2712-2718.
15. Elkeles, R.S., Feher, M.D., Flather, M.D., Godsland, I.F., Nugara, F., Richmond, W., Rubens, M.B., and Wang, D. (2004). The association of coronary calcium score and conventional cardiovascular risk factors in Type 2 diabetic subjects asymptomatic for coronary heart disease (The PREDICT Study). *Diabet. Med.* **21**, 1129-1134.
16. Weisberg, S.P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R.L., and Ferrante, A.W., Jr. (2003). Obesity is associated with macrophage accumulation in adipose tissue. *J. Clin. Invest.* **112**, 1796-1808.
17. Hotamisligil, G.S., Arner, P., Caro, J.F., Atkinson, R.L., and Spiegelman, B.M. (1995). Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. *J. Clin. Invest.* **95**, 2409-2415.

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18. Kern, P.A., Saghizadeh, M., Ong, J.M., Bosch, R.J., Deem, R., and Simsolo, R.B. (1995). The expression of tumor necrosis factor in human adipose tissue. Regulation by obesity, weight loss, and relationship to lipoprotein lipase. *J. Clin. Invest.* **95**, 2111-2119.
19. Berg, A.H., and Scherer, P.E. (2005). Adipose tissue, inflammation, and cardiovascular disease. *Circ. Res.* **96**, 939-949.
20. Rocha, V.Z., and Libby, P. (2009). Obesity, inflammation, and atherosclerosis. *Nat. Rev. Cardiol.* **6**, 399-409.
21. Medzhitov, R. (2001). Toll-like receptors and innate immunity. *Nat. Rev. Immunol.* **1**, 135-145.
22. Edfeldt, K., Swedenborg, J., Hansson, G.K., and Yan, Z.Q. (2002). Expression of toll-like receptors in human atherosclerotic lesions: a possible pathway for plaque activation. *Circulation.* **105**, 1158-1161.
23. Kazemi, M.R., McDonald, C.M., Shigenaga, J.K., Grunfeld, C., and Feingold, K.R. (2005). Adipocyte fatty acid-binding protein expression and lipid accumulation are increased during activation of murine macrophages by toll-like receptor agonists. *Arterioscler. Thromb. Vasc. Biol.* **25**, 1220-1224.
24. Liu, X., Ukai, T., Yumoto, H., Davey, M., Goswami, S., Gibson, F.C., 3rd, and Genco, C.A. (2008). Toll-like receptor 2 plays a critical role in the progression of atherosclerosis that is independent of dietary lipids. *Atherosclerosis.* **196**, 146-154.
25. Schoneveld, A.H., Hoefler, I., Sluijter, J.P., Laman, J.D., de Kleijn, D.P., and Pasterkamp, G. (2008). Atherosclerotic lesion development and Toll like receptor 2 and 4 responsiveness. *Atherosclerosis.* **197**, 95-104.
26. Vink, A., Schoneveld, A.H., van der Meer, J.J., van Middelaar, B.J., Sluijter, J.P., Smeets, M.B., Quax, P.H., Lim, S.K., Borst, C., Pasterkamp, G., and de Kleijn, D.P. (2002). In vivo evidence for a role of toll-like receptor 4 in the development of intimal lesions. *Circulation.* **106**, 1985-1990.
27. Ishikawa, Y., Satoh, M., Itoh, T., Minami, Y., Takahashi, Y., and Akamura, M. (2008). Local expression of Toll-like receptor 4 at the site of ruptured plaques in patients with acute myocardial infarction. *Clin. Sci. (Lond).* **115**, 133-140.
28. Versteeg, D., Hoefler, I.E., Schoneveld, A.H., de Kleijn, D.P., Busser, E., Strijder, C., Emons, M., Stella, P.R., Doevendans, P.A., and Pasterkamp, G. (2008). Monocyte toll-like receptor 2 and 4 responses and expression following percutaneous coronary intervention: association with lesion stenosis and fractional flow reserve. *Heart.* **94**, 770-776.
29. Liuzzo, G., Angiolillo, D.J., Buffon, A., Rizzello, V., Colizzi, C., Ginnetti, F., Biasucci, L.M., and Maseri, A. (2001). Enhanced response of blood monocytes to in vitro lipopolysaccharide-challenge in patients with recurrent unstable angina. *Circulation.* **103**, 2236-2241.
30. Methe, H., Kim, J.O., Kofler, S., Weis, M., Nabauer, M., and Koglin, J. (2005). Expansion of circulating Toll-like receptor 4-positive monocytes in patients with acute coronary syndrome. *Circulation.* **111**, 2654-2661.
31. Verhoeven, B.A., Velema, E., Schoneveld, A.H., de Vries, J.P., de Bruin, P., Seldenrijk, C.A., de Kleijn, D.P., Busser, E., van der Graaf, Y., Moll, F., and Pasterkamp, G. (2004). Athero-express: differential atherosclerotic plaque expression of mRNA and protein in relation to cardiovascular events and patient characteristics. Rationale and design. *Eur. J.Epidemiol.* **19**, 1127-1133.
32. Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K., and Akira, S. (1999). Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J. Immunol.* **162**, 3749-3752.
33. Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K., and Akira, S. (1999). Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity.* **11**, 443-451.
34. Visser, M., Bouter, L.M., McQuillan, G.M., Wener, M.H., and Harris, T.B. (1999). Elevated C-reactive protein levels in overweight and obese adults. *JAMA.* **282**, 2131-2135.
35. Arnaout, M.A. (1990). Structure and function of the leukocyte adhesion molecules CD11/CD18. *Blood.* **75**, 1037-1050.

36. Zhang, W.Y., Schwartz, E., Wang, Y., Attrep, J., Li, Z., and Reaven, P. (2006). Elevated concentrations of nonesterified fatty acids increase monocyte expression of CD11b and adhesion to endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **26**, 514-519.
37. Kawakami, A., Osaka, M., Aikawa, M., Uematsu, S., Akira, S., Libby, P., Shimokado, K., Sacks, F.M., and Yoshida, M. (2008). Toll-like receptor 2 mediates apolipoprotein CIII-induced monocyte activation. *Circ. Res.* **103**, 1402-1409.
38. Shi, H., Kokoeva, M.V., Inouye, K., Tzameli, I., Yin, H., and Flier, J.S. (2006). TLR4 links innate immunity and fatty acid-induced insulin resistance. *J. Clin. Invest.* **116**, 3015-3025.
39. Konner, A.C., and Bruning, J.C. (2011). Toll-like receptors: linking inflammation to metabolism. *Trends Endocrinol. Metab.* **22**, 16-23.
40. Weyrich, P., Staiger, H., Stancakova, A., Machicao, F., Machann, J., Schick, F., Stefan, N., Kuusisto, J., Laakso, M., Schafer, S., Fritsche, A., and Haring, H.U. (2010). The D299G/T399I Toll-like receptor 4 variant associates with body and liver fat: results from the TULIP and METSIM Studies. *PLoS One.* **5**, e13980.
41. Schwartz, E.A., Zhang, W.Y., Karnik, S.K., Borwege, S., Anand, V.R., Laine, P.S., Su, Y., and Reaven, P.D. (2010). Nutrient modification of the innate immune response: a novel mechanism by which saturated fatty acids greatly amplify monocyte inflammation. *Arterioscler. Thromb. Vasc. Biol.* **30**, 802-808.
42. Arita, Y., Kihara, S., Ouchi, N., Takahashi, M., Maeda, K., Miyagawa, J., Hotta, K., Shimomura, I., Nakamura, T., Miyaoaka, K., Kuriyama, H., Nishida, M., Yamashita, S., Okubo, K., Matsubara, K., Muraguchi, M., Ohmoto, Y., Funahashi, T., and Matsuzawa, Y. (1999). Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem. Biophys. Res. Commun.* **257**, 79-83.
43. Yamaguchi, N., Argueta, J.G., Masuhiro, Y., Kagishita, M., Nonaka, K., Saito, T., Hanazawa, S., and Yamashita, Y. (2005). Adiponectin inhibits Toll-like receptor family-induced signaling. *FEBS Lett.* **579**, 6821-6826.
44. Wulster-Radcliffe, M.C., Ajuwon, K.M., Wang, J., Christian, J.A., and Spurlock, M.E. (2004). Adiponectin differentially regulates cytokines in porcine macrophages. *Biochem. Biophys. Res. Commun.* **316**, 924-929.
45. Chen, H., Cowan, M.J., Hasday, J.D., Vogel, S.N., and Medvedev, A.E. (2007). Tobacco smoking inhibits expression of proinflammatory cytokines and activation of IL-1R-associated kinase, p38, and NF-kappaB in alveolar macrophages stimulated with TLR2 and TLR4 agonists. *J. Immunol.* **179**, 6097-6106.

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Table 1

Patient characteristics. Number of patients together with the percentage of patients, depicted between the brackets, are provided for risk factors and medication. In both groups obese patients (BMI > 25) were significantly younger compared with non-obese patients.

PCI study = percutaneous coronary intervention study, CEA study = carotid endarterectomy study

* p < 0.05 ** p < 0.001

† from 18 patients no data could be obtained concerning family history of heart disease.

Table 2

TNF-alpha levels following Toll like receptor (TLR) 2 stimulation (with Pam3Cys) and TLR 4 stimulation (with LPS) and risk factors for atherosclerosis. Levels (pg/ml) are depicted as median values with interquartile range (IQR). Number of patients are provided in table 1.

PCI study = percutaneous coronary intervention study, CEA study = carotid endarterectomy study

* p < 0.05 ** p < 0.010

† from 18 patients no data could be obtained concerning family history of heart disease. These patients were excluded for this analysis.

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Figure 1

Toll like receptor (TLR) response and obesity: TNF-alpha levels (ln transformed) are depicted on the y-axis with levels following TLR2 stimulation (with Pam3Cys) at the left and levels following TLR4 stimulation (with LPS) at the right. The grey circles represent obese patients, the white circles are non-obese patients. P-values are adjusted for cardiovascular risk factors.

PCI study = percutaneous coronary intervention study, CEA study = carotid endarterectomy study

Figure 2

CD11b expression and obesity. CD11b expression (mean fluorescence intensity, ln transformed) on CD14+ leucocytes is depicted on the y-axis for non-obese (grey) and obese patients (white). The upper left figure shows the results at baseline for the PCI study (A), the upper right figure shows the results at baseline for the CEA study (B). Lower figures show CD11b expression following TLR2 (C), TLR4 (D) and PMA (E) stimulation. F shows an example of one patient with CD14+ monocytes. P-values are adjusted for cardiovascular risk factors.

PCI study = percutaneous coronary intervention study; CEA study = carotid endarterectomy study
TLR = Toll like receptor; PMA = Phorbol 12-myristate 13-acetate

Figure 3

High sensitive C-reactive protein (hsCRP) levels were measured in the PCI study. hsCRP levels (ln transformed) are depicted on the y-axis with the BMI groups depicted on the x-axis with non-obese vs obese depicted at the left and three BMI categories depicted at the right. P-values are adjusted for cardiovascular risk factors.

PCI study = percutaneous coronary intervention study; CEA study = carotid endarterectomy study

Figure 4

Toll like receptor (TLR) response and obesity: IL6 and IL8 levels were determined in the CEA study following 2 hours of whole blood Toll Like Receptor stimulation. IL6 levels (ln transformed) are depicted on the left y-axis and IL-8 levels are depicted at the right y-axis following TLR2 stimulation (with Pam3Cys) at the left and following TLR4 stimulation (with LPS) at the right. The grey circles represent obese patients, the black circles are non-obese patients. P-values are adjusted for cardiovascular risk factors.

Characteristic	PCI study Total (78)	BMI < 25 (23)	BMI > 25 (55)	CEA study Total (104)	BMI < 25 (37)	BMI > 25 (67)
Sex	56 ♂ 22 ♀	15♂ 8♀	41♂ 14♀	69♂ 35♀	23 ♂ 14 ♀	46♂ 21♀
Age (years) median [IQR]	62.5[52.5-69.7]	65.0[61.0-72.0]*	58.6[51.9-67.2]*	70.4[62.2-81.0]	73.7[66.3-79.9]*	69.3[61.7-74.6]*
BMI (kg/m ²) median [IQR]	26.9[24.6-34.9]	24.0[22.6-24.7]**	28.7[26.6-32.4]**	26.2[24.1-29.7]	23.1[21.8-24.2]**	29.0[26.6-30.7]**
Hypertension	52 (67%)	13 (57%)	39 (71%)	87 (84%)	30 (81%)	57 (85%)
Diabetes	16 (21%)	4 (17%)	12 (22%)	21 (20%)	8 (22%)	13 (19%)
Hypercholesterolemia	72 (92%)	21 (91%)	51 (93%)	83 (80%)	28 (76%)	55 (82%)
Smoking (yes)	5 (6%)	2 (9%)	3 (6%)	26 (25%)	7 (19%)	19 (28%)
Family history of heart disease < 60 years	41 (53%)	13 (57%)	28 (51%)	27 (31%) [†]	8 (30%) [†]	19 (32%) [†]
Medication						
Beta-blocker	73(94%)	23 (100%)	50 (91%)	50 (48%)	16 (43%)	34 (51%)
Calcium-antagonist	24 (31%)	6 (26%)	18 (33%)	24 (23%)	8 (22%)	16 (24%)
Nitrates	37 (47%)	10 (44%)	27 (49%)	11 (11%)	4 (11%)	7 (10%)
ACE-inhibitor	19 (24%)	4 (17%)	15 (27%)	33 (32%)	10 (27%)	23 (34%)
A2-antagonist	10 (13%)	4 (17%)	6 (11%)	8 (8%)	3 (8%)	5 (8%)
Diuretics	10 (13%)	2 (9%)	8 (15%)	46 (44%)	13 (35%)	33 (49%)
Anti-arythmic	5 (6%)	2 (9%)	3 (6%)	4 (4%)	1 (3%)	3 (5%)
Statin	72 (92%)	21 (91%)	51 (93%)	84 (81%)	28 (76%)	56 (84%)
Asasantin	69 (89%)	20 (87%)	49 (89%)	62 (60%)	21 (57%)	41 (61%)
Clopidogel	60 (77%)	19 (83%)	41 (75%)	7 (7%)	4 (11%)	3 (4%)
Coumarin	8 (10%)	4 (17%)	4 (7%)	7 (7%)	4 (11%)	3 (4%)

Table 1

TLR2 stimulation (TNF-alpha pg/ml)	PCI study		CEA study	
	present	not present	present	not present
Hypertension	11 [3 – 35]	18 [8 – 50]	12 [6 – 22]	14 [7 – 25]
Diabetes	10 [1 – 26]	14 [6 – 44]	12 [7 – 20]	12 [7 – 25]
Hypercholesterolemia	12 [4 – 42]	23 [8 – 40]	11 [6 – 22]	15 [9 – 31]
Family history of heart disease < 60 years	15 [6 – 46]	11 [3 – 36]	13 [7 – 32] [†]	12 [6 – 19] [†]
BMI (BMI < 25 - BMI > 25)	7 [2 – 19]**	17 [8 – 49]**	9 [6 – 16]*	15 [8 – 28]*
Smoking (yes - no)	8 [2 – 22]	13 [5 – 43]	11 [6 – 20]	13 [7 – 25]
Active smoker - Passed smoker < 6 months	8 [2 – 22]	11 [4 – 50]	11 [6 – 20]	7 [4 – 23]
Passed smoker > 6 months - No smoking ever	14 [3 – 43]	13 [5 – 45]	13 [9 – 22]	16 [10 – 41]
TLR4 stimulation (TNF-alpha pg/ml)				
Hypertension	221 [113 – 328]	230 [143 – 317]	175 [102 – 297]	245 [152 – 384]
Diabetes	191 [79 – 218]	239 [128 – 334]	196 [112 – 271]	192 [110 – 317]
Hypercholesterolemia	223 [113 – 328]	219 [196 – 287]	190 [103 – 302]	202 [146 – 329]
Family history of heart disease < 60 years	217 [114 – 311]	222 [142 – 364]	227 [112 – 340] [†]	165 [108 – 297] [†]
BMI (BMI < 25 - BMI > 25)	181 [91 – 237]*	257 [129 – 369]*	154 [105 – 261]	209 [119 – 340]
Smoking (yes - no)	94 [54 – 268]	224 [127 – 325]	206 [125 – 312]	185 [111 – 300]
Active smoker - Passed smoker < 6 months	94 [54 – 268]	257 [168 – 363]	206 [125 – 312]	163 [68 – 303]
Passed smoker > 6 months - No smoking ever	213 [115 – 314]	243 [159 – 359]	215 [115 – 300]	191 [117 – 316]

Table 2

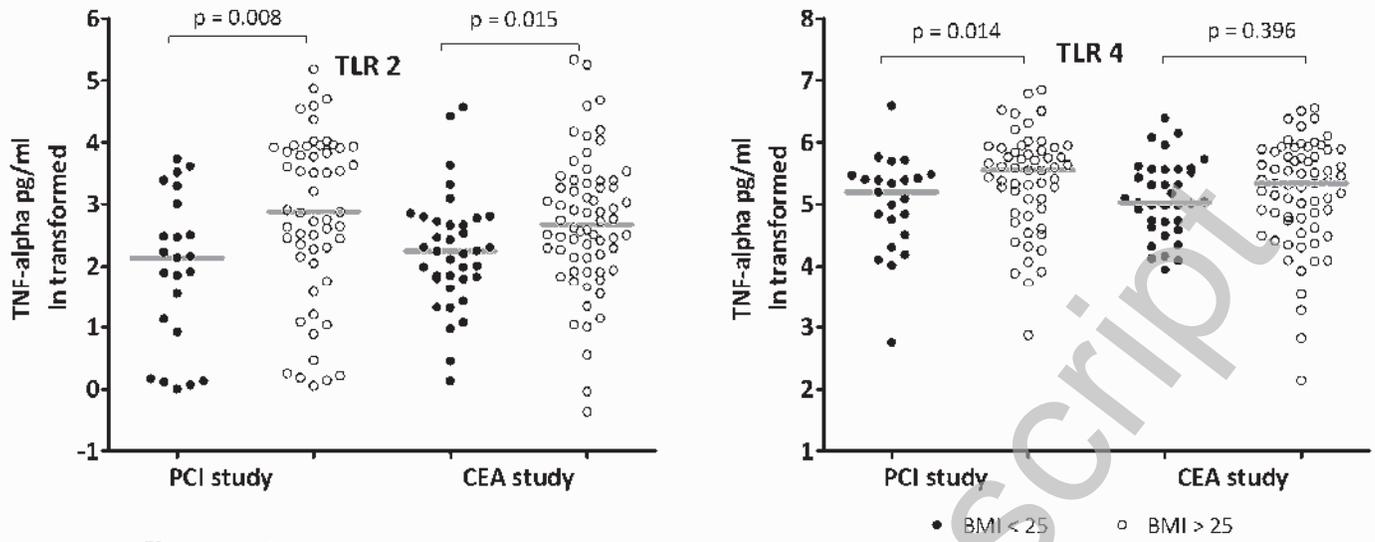


figure 1

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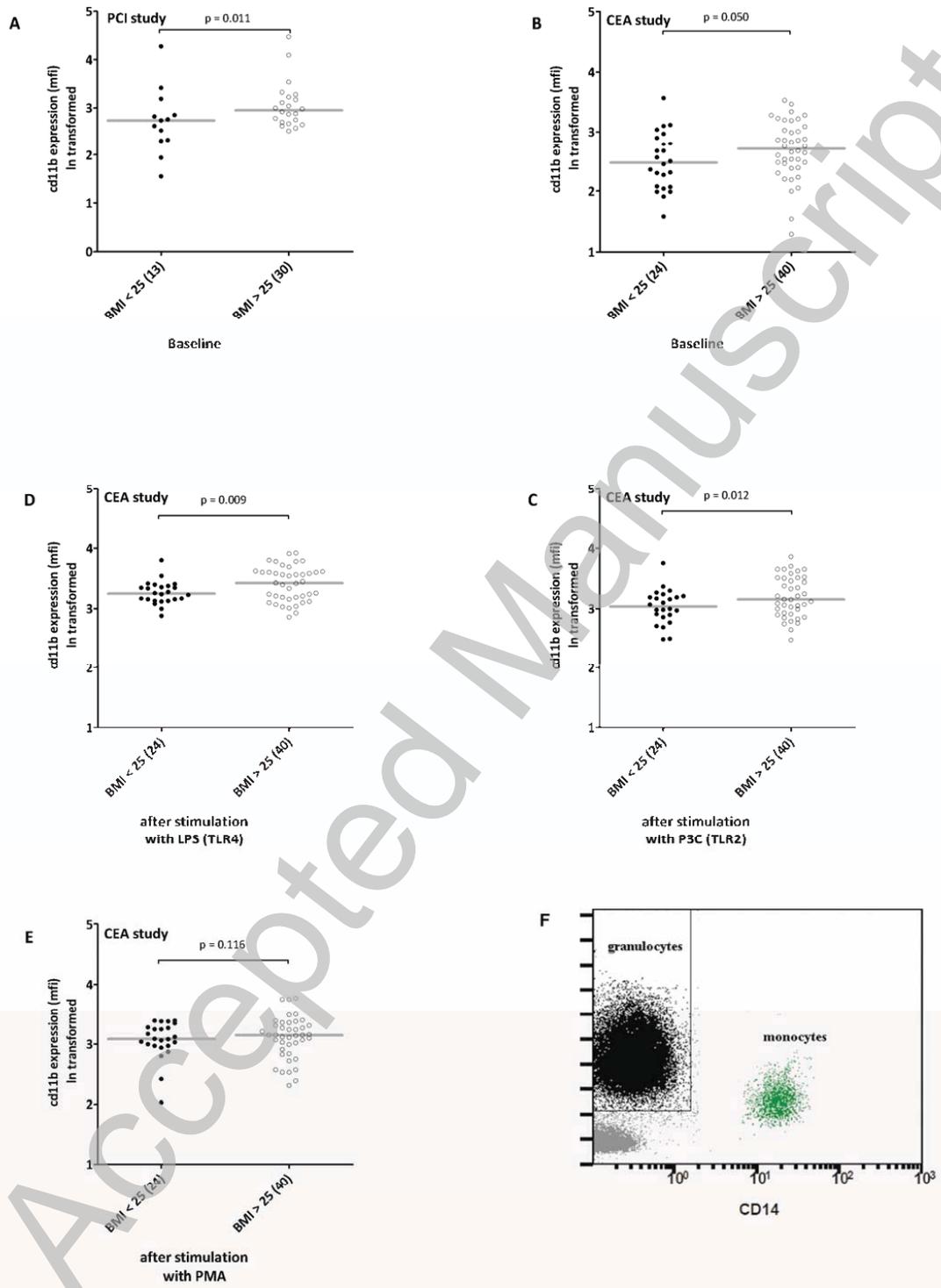


figure 2

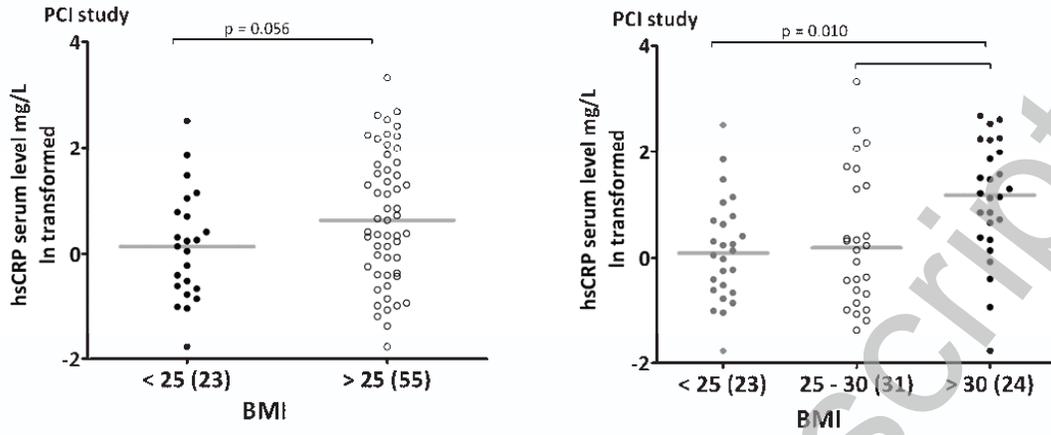


figure 3

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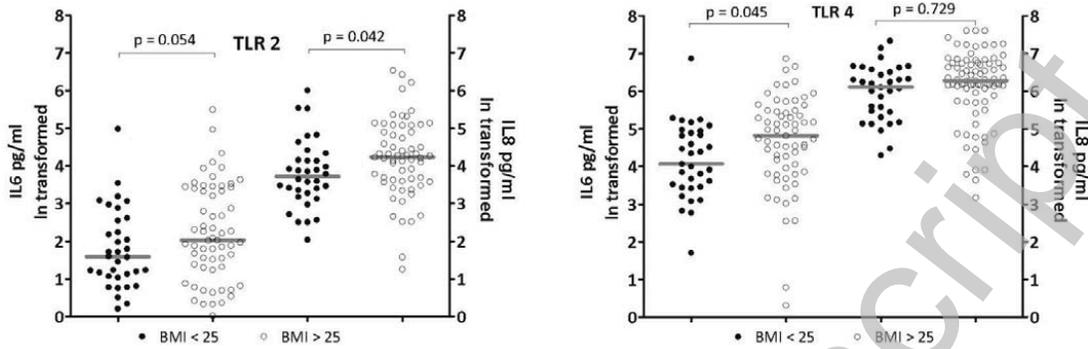


figure 4

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