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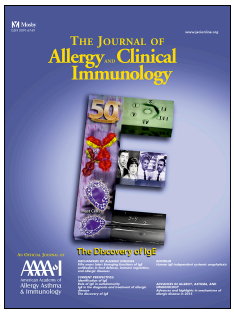

Saturated fatty acids, obesity and the NLRP3 inflammasome in asthma

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Abstract

Background: Both obesity and high dietary fat intake activate the NLRP3 inflammasome.

Objective: We aimed to examine NLRP3 inflammasome activity in the airways of obese asthmatics, following macronutrient overload and in immune cells challenged by inflammasome triggers.

Methods: *Study 1*: Cross-sectional, observational study of non-obese (n=51) and obese (n=76) asthmatic adults. *Study 2:* Randomized, crossover, acute feeding study in 23 asthmatic adults (n=12 non-obese, n=11 obese). Subjects consumed 3 isocaloric meals on 3 separate occasions: saturated fatty acid (SFA), n-6 polyunsaturated (PUFA) and carbohydrate (CHO); and were assessed at 0 and 4 hours. For Study 1 and 2, airway inflammation was measured using sputum differential cell counts, IL-1β protein (ELISA) and sputum cell gene expression (Nanostring nCounter). *Study 3:* Peripheral blood neutrophils and monocytes were isolated using Ficoll density gradient and magnetic bead separation, and incubated with or without palmitic acid, LPS or TNFα for 24 hours and IL-1β release measured (ELISA).

Results: *Study 1*: NLRP3 and NOD1 gene expression were upregulated, and sputum IL-1β protein levels higher, in obese versus non-obese asthmatics. *Study 2*: The SFA meal led to increases in sputum %neutrophils and sputum cell gene expression of TLR4 and NLRP3 at 4 hours, in non-obese asthmatics. *Study 3*: Neutrophils and monocytes released IL-1β when challenged with a combination of palmitic acid and LPS or TNFα.

Conclusion: The NLRP3 inflammasome is a potential therapeutic target in asthma. Behavioural interventions that reduce fatty acid exposure, such as weight loss and dietary saturated fat restriction warrant further exploration.

Clinical implications: Both obesity and saturated fat intake cause NLRP3 inflammasome-mediated airway inflammation in asthma. Hence weight loss and dietary fat restriction warrant further exploration as anti-inflammatory strategies in asthma.

Capsule summary: The NLRP3 inflammasome is upregulated in obese asthmatics and following a high saturated fat meal in non-obese asthmatics. Both reversal of obesity and restriction of dietary saturated fat intake warrant further exploration as anti-inflammatory strategies in asthma.

Key words: fatty acids; saturated fat; obesity; inflammasome; airway inflammation; interleukin 1 beta; asthma

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Abbreviations:

ANOVA, analysis of variance
BMI, body mass index
C10:0, capric acid
C14:0, myristic acid
C16:0, palmitic acid
C18:0, stearic acid
C18:2 n-6, linoleic acid
C20:4 n-6, arachidonic acid
CD, cluster of differentiation
CHO, carbohydrate
CXCL8, CXC chemokine ligand 8
EDTA, ethylenediaminetetraacetic acid
ELISA, enzyme linked immunosorbent assay
ER, endoplasmic reticulum
FA, fatty acid
FEV1, forced expiratory volume in 1 second
FVC, forced vital capacity
IL, interleukin
IL1-R, IL-1 receptor
IL-1RA, IL-1 receptor antagonist
IL-1RAP, IL-1 receptor accessory protein
IL-1RN, IL-1 receptor agonist
LPS, lipopolysaccharide
MUFA, monounsaturated fatty acid
NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells
NLR, nod like receptor
NLRP3, nucleotide oligomerisation domain-like receptor protein 3
NOD, nucleotide oligomerisation domain
PGK1, phosphoglycerate kinase 1
PUFA, polyunsaturated fatty acids
RNA, ribonucleic acid
SFA, saturated fatty acid
STRING, Search Tool for the Retrieval of Interacting Genes
TLR, toll like receptor toll like receptor
TNF, tumor necrosis factor

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Introduction

Asthma is a chronic inflammatory airways disease which affects 300 million people worldwide, with prevalence rates of >10% in many westernized countries (1). Obesity rates are also alarmingly high, with over 20% of adults in developed countries estimated to be obese (2). Obesity increases asthma risk and 'obese asthma' is recognised as a distinct clinical phenotype (3) that is difficult to manage, characterised by worse lung function (4) and symptoms (5) and reduced response to asthma pharmacotherapy, including glucocorticoids (6-8). The unique inflammatory profile induced by excess adipose tissue in obesity likely contributes to this phenotype (8).

In obesity, adipocytes and adipose tissue-resident macrophages release pro-inflammatory mediators, such as tumour necrosis factor (TNFα) and interleukin (IL)-6, leading to chronic systemic inflammation. Integral to this process is the activation of the nucleotide oligomerisation domain (NOD)-like receptor protein 3 (NLRP3)(9). NLRP3 can be activated by excess SFAs, cholesterol and cellular debris following adipocyte apoptosis, leading to the assembly of the NLRP3 inflammasome, that recruits and activates caspase-1 (Casp1), leading to the secretion of IL-1β (9). We (10) and others (11, 12), have described increased airway neutrophilia in obese asthma. Whether NLRP3 inflammasome activation contributes to neutrophil influx in obese asthma is unknown.

Independent of the effects of excess adipose tissue, macronutrient loading, which is common in obese individuals, induces postprandial inflammation, *via* mechanisms such as direct activation of innate immune receptors and endoplasmic reticulum (ER) stress (13). We have previously shown that a high-energy fast food meal induces airway neutrophilia and upregulates sputum cell toll like receptor 4 (TLR4) gene expression in asthma (14). However, the possible role of NLRP3 inflammasome activity in postprandial airway inflammation has not been determined. Furthermore, the contribution of different macronutrients to postprandial airway inflammation has not been examined. Addressing these key questions will provide a better understanding of the nature of airway inflammation in obese asthma, which will enable the development of more effective treatment strategies for this subgroup of asthmatics.

The aims of this study were to examine: 1) the activity of the NLRP3 inflammasome in obese asthmatic airways; 2) the effect of macronutrient (SFA, n-6 PUFA and CHO) overload on inflammation and NLRP3 inflammasome activity in asthmatic airways and; 3) the effect of SFAs on NLRP3 inflammasome activity in specific immune cells (neutrophils and monocytes).

Materials and Methods

Study 1: Obese versus non-obese asthmatics: cross sectional comparison of inflammatory pathways

A cross-sectional, observational study was conducted in 127 adult asthmatics, categorised as non-obese (BMI<30 kg/m^2; n=51) or obese (BMI ≥30 kg/m^2; n=76). Data from a subset of these subjects has been previously reported (14-16). Subjects fasted overnight and asthma medications were withheld (short acting bronchodilators, 6 hrs; long acting bronchodilators and inhaled corticosteroids, 24 hrs). Blood was collected, spirometry and sputum induction were performed during hypertonic saline challenge (17).

Study 2: Acute meal challenge effects on asthmatic airways: SFA and n-6 PUFA versus CHO

A randomized, crossover trial was conducted in 23 adults (n=12 non-obese and n=11 obese) with stable asthma. Prior to each visit, subjects fasted overnight and asthma medications were withheld (as above). At 0 hours, blood was collected and sputum induced (as above)(17), then the study meal was consumed. At 4 hours, blood and induced sputum were collected again. On the following visits, subjects repeated these procedures with an alternate study meal. Meals were consumed in random order with a minimum washout period of 7 days between visits.

Three different isocaloric meals were tested, rich in either SFA, n-6 PUFA or CHO. At 0 hours, subjects consumed 200g potato. Subjects also consumed double cream and butter (SFA meal), safflower oil (n-6 PUFA meal), or glucose confectionary (CHO meal). Meals were timed to ensure peak nutrient concentrations at 4 hours; 0 hours for the FA meals and 2 hours for the CHO meal. The nutrient composition of the meals is described in Table 1. Study 1 and 2 were approved by the Hunter New England and University of Newcastle Human Research Ethics Committees. Written informed consent was obtained from all subjects. Study 2 was prospectively registered with the Australian and New Zealand Clinical Trials Registry (ACTRN12612000697886).

Procedures

Subject Characterization

Subjects were recruited from ambulatory care clinics at John Hunter Hospital, Newcastle, Australia. Asthma was defined by clinical history and airway hyper-responsiveness to hypertonic saline (4.5%), defined as ≥15% fall in forced expiratory volume in 1 second (FEV_1) from baseline. Stable asthma was defined as no exacerbation, respiratory tract infection or oral corticosteroid use in the past 4 weeks. Skin prick allergy testing determined atopic status.

Blood collections and processing

Blood was collected into EDTA tubes and full blood counts were performed using a Beckman Coulter LH series analyzer (Beckman Coulter Ltd, Brea, CA, USA) by Hunter Area Pathology Service (Newcastle, Australia). In addition, plasma was separated by centrifugation (4°C, 10min, 3,000g) and stored at -80°C for FA analysis as described previously (18), using gas chromatography with a 30m x 0.25mm (DB-225) fused carbon-silica column, coated with cyanopropylphenyl (J & W Scientific, Folsom, CA) and flame ionisation detector (Hewlett Packard 6890 Series Gas Chromatograph with Chemstations software, version A.04.02, Hewlett-Packard, Palo Alto, CA).

Sputum collection and processing

Lower respiratory sputum portions were selected and dispersed using dithiothreitol as previously described (17). Total cell counts and viability were performed by haemocytometer and cytospins used for differential cell counts. Sputum supernatant concentrations of IL-1β were measured using ELISA Duoset (R&D Systems, Minneapolis, USA), validated for use in sputum (19). For gene expression analysis, 100 µL of selected sputum was added to Buffer RLT (Qiagen, Hilden, Germany) and stored at -80°C for RNA extraction.

Nanostring analysis

RNA was extracted from sputum using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and quantitated using the Quant-iT RiboGreen RNA Quantitation Assay Kit (Molecular Probes Inc., Life Technologies, Carlsbad, CA). Using the Nanostring nCounter Analysis System (Nanostring Technologies, Seattle, WA), gene expression was analyzed using a custom-designed codeset containing 249 inflammation-related genes with 6 reference genes.

Study 3: In vitro investigation of the effects of SFAs on neutrophils and monocytes

Peripheral blood neutrophil and monocyte isolation

Peripheral blood was collected from healthy volunteers and neutrophils and monocytes isolated. Blood collected in citrate dextrose tubes was mixed with 10% dextran (MP Biomedicals, Santa Ana, California, USA), the top layer was overlayed on Ficoll Paque-Plus density gradient medium (GE Healthcare, Little Chalfont, UK) and centrifuged (10 min, 2000g). Mononuclear cells at the interface of plasma and the Ficoll layer were collected and monocytes isolated using CD14+ magnetic beads. Red blood cells were lysed and neutrophils were positively selected using CD16+ magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany)(20). Neutrophils and monocytes were resuspended in RPMI 1640 containing 1% HEPES (Life Technologies, Mulgrave, VIC, Australia), 1% FBS and 1% Antibiotic-Antimycotic and seeded (1 x 10^6 cells/mL).

Treatment of cells

Cells were treated with palmitic acid (C16:0). Stock solutions of 0.5M palmitic acid (Sigma Aldrich, Missouri, USA) were prepared in 100% ethanol and stored at -20ºC. Working solutions of 10mM were generated by incubating palmitic acid in FA-free BSA (Sigma Aldrich)(5:1) at 65ºC for 10 min, then 39ºC for 90 min with occasional vortexing. Neutrophils and monocytes were stimulated with 0 or 100µM palmitic acid and vehicle (EtOH/BSA/cell culture medium), with or without LPS (1µg/mL)(Sigma Aldrich) or TNFα (1ng/mL) (Sigma Aldrich), then incubated at 37°C with 5% CO2 for 24h. Cell-free supernatants were stored at -80°C for measurement of IL-1β by ELISA (R&D Systems, Minnesota, USA).

Statistical analysis

Analysis was conducted using Stata 11 (Stata Corp, College Station, TX). Parametric and non-parametric data are presented as mean ± standard deviation (SD) and median (quartile 1, quartile 3) respectively. Comparisons were conducted using Students t-test, Mann Whitney U test or Wilcoxon signed rank test for quantitative data and Chi-squared or Fisher's exact test for frequency data. Associations between variables were examined using Pearson's correlation coefficient for parametric data and Spearman's rank correlation coefficient for non-parametric data. For the acute meal study (Study 2), changes within intervention group compared to baseline were compared using Wilcoxon signed rank test. Differences between intervention groups were compared using General Linear Models with atopy as a covariate. Nanostring data was analyzed using nSolver Analysis Software v2.5 (Nanostring Technologies, Seattle, USA). Raw counts were normalized to 6 positive controls and the reference gene, phosphoglycerate kinase 1 (PGK1) and log transformed. Genes with a fold change >1.5, were assessed using unpaired (Study 1) or paired (Study 2) t-tests using the Benjamini Hochberg adjustment for multiple comparisons (false discovery rate 0.25). Search Tool for the Retrieval of Interacting Genes, STRING v10 (http://string-db.org)(21) was used to investigate pathway interactions between differentially expressed

genes, using medium confidence scores >0.4. In Study 1, Nanostring gene expression analysis was performed in a subset of obese (n=11) and non-obese (n=14) asthmatics. In Study 2, Nanostring analysis was performed in the non-obese subjects for whom paired sputum samples were available from both 0 and 4 hours after the SFA meal (n=4).

Results

Obese versus non-obese asthmatics: cross sectional comparison of inflammatory pathways

Subject characteristics are described in Table 2. Obese asthmatics had higher plasma levels of total FAs, saturated FAs, monounsaturated FAs, C16:0, C18:0 and C20:4n-6, compared to non-obese asthmatics. Nanostring analysis of sputum cell gene expression in obese versus non-obese asthmatics, identified 13 genes with >1.5 fold change that were differentially expressed (Table 3). The most significantly upregulated gene in obese asthma was IL-5, followed by NOD1. IL-5 expression was positively associated with NLRP3 expression (r=0.483, p=0.015).

As NLRP3 gene expression was upregulated in obese asthmatics, we measured sputum IL-1β concentrations, a marker of NLRP3 activity. Sputum IL-1β concentrations were higher in obese versus non-obese asthmatics (Figure 1). Sputum cell NLRP3 gene expression and sputum IL-1β protein concentrations correlated with BMI (Figure 2a and 2b). Sputum cell NLRP3 gene expression correlated with sputum IL-1β protein concentrations (Figure 2c). TLR4 gene expression correlated with BMI (r=0.448, p=0.025) and sputum %neutrophils (r=0.435, p=0.030). When analyzed by gender, in females only (n=14), IL-1β gene expression correlated with NLRP3 gene expression (r=0.877, p<0.001), TLR4 gene expression (r=0.697, p=0.007) and sputum %neutrophils (r=0.775, p=0.003) and inversely correlated with %predicted FEV1 (r=-0.649, p=0.014) and %predicted FVC (r=-0.613, p=0.022).

Acute meal challenge effects on asthmatic airways: SFA and n-6 PUFA versus carbohydrate

Subject characteristics are described in Table 4. At 4 hours after the n-6 PUFA meal challenge, plasma n-6 PUFA increased compared to baseline (total n-6 PUFA: +11.9%, p=0.005; 18:2n-6: +16%, p=0.003). At 4 hours after the SFA meal challenge, plasma SFA increased compared to baseline (total SFA: +11.9%, p=0.005; C16:0: +20.9%, p=0.001; C18:0: +19.7%, p=0.044). Following the carbohydrate meal, there were no changes in plasma FA concentrations.

Changes in inflammatory cell counts following each intervention

Changes in blood and induced sputum leukocyte counts following each intervention are described in Table 5. In blood, total white cell, lymphocyte and monocyte counts increased in each intervention group at 4 hours compared to baseline. Blood neutrophils increased following the fat meals (SFA and n-6 PUFA) but not the carbohydrate meal. The increases in white cell and neutrophil counts were higher following the SFA meal than the carbohydrate meal. In induced sputum, total cell and macrophage counts decreased at 4 hours versus baseline following both the carbohydrate and n-6 PUFA meals. An increase in eosinophils was evident in the n-6 PUFA group only.

When analysed according to BMI category, in blood from both non-obese and obese asthmatics, total white cell and neutrophil counts were increased at 4 hours compared to baseline after the fat meals (SFA and n-6 PUFA) (Figure 3a). In the non-obese group, the change in white cells and neutrophils was higher following the fat meals than the carbohydrate meal (Figure 3a).

In sputum from non-obese subjects, %neutrophils increased following the SFA meal only (Figure 3b). Total cell counts decreased after the carbohydrate and n-6 PUFA meals, but not the SFA meal, at 4 hours compared with baseline (Figure 3b). In sputum from obese subjects, the only significant change was a decrease in total cell count following the carbohydrate meal at 4 hours compared to baseline (Figure 3b).

Effect of SFA on sputum cell gene expression in non-obese asthmatics

Nanostring analysis in sputum from non-obese subjects, identified 9 genes with >1.5 fold change that were differentially expressed at 4 hours after the SFA meal compared to baseline (Table 6). From these genes a main gene network containing 8 genes was identified (Figure 4).

***In vitro* investigation of SFA effects on neutrophils and monocytes**

In both neutrophils and monocytes, *ex vivo* exposure to palmitic acid alone did not induce IL-1β release. However, in both cell types, the combination of palmitic acid and LPS or TNFα, led to increased IL-1β release (Figure 5).

Discussion

This study provides important new insight into airway NLRP3 inflammasome activity in asthmatics with a high BMI and following excess macronutrient intake. We have shown increased circulating FA levels, activation of the innate immune receptor, NOD1, increased NLRP3 activity and IL-1β protein levels in obese asthmatics. We have also determined that SFAs induce postprandial airway inflammation with increased TLR4, NLRP3 and IL-1 pathway gene expression, in non-obese asthmatics. Using *in vitro* models, we have confirmed that neutrophils and monocytes, are a key source of NLRP3 inflammasome-driven postprandial inflammation. Hence, our studies describe the independent, yet consistent effects of both a high BMI and high saturated fat intake, on NLRP3 inflammasome activation in asthma, with both contributing to airway inflammation.

We identified 13 genes that were differentially expressed in obese versus non-obese asthmatics. This included NOD1 and NLRP3, which are both members of the nod-like receptor (NLR) family and involved in the activation of the NLRP3 inflammasome. The NLRP3 inflammasome integrates metabolic and inflammatory processes, stimulated by various metabolites, including fatty acids, to induce production of IL-1β, in a two-step process (22). In the first step, triggers such as SFAs or TNFα, bind to innate immune receptors such as TLR4, NOD1, or TNFR1/2, inducing nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) activity and production of pro-IL-1β. In the second step, a stress signal, such as ATP or ceramides (which can be produced from SFAs (23)), triggers a signalling cascade that mediates cleavage of procaspase-1 to active caspase, then cleavage of pro-IL-1β to mature IL-1β (22). Our *ex vivo* experiments demonstrate the two-stage process by which NLRP3 inflammasome activation occurs. Free saturated fatty acid exposure (palmitic acid)

alone did not stimulate inflammation. However, when palmitic acid was combined with another stimuli known to be associated with the obese state, ie LPS or TNFα, IL-1β release was induced from peripheral blood inflammatory cells. Indeed, elevated plasma fatty acid levels and increased NOD1 and NLRP3 inflammasome expression in adipose tissue are reflective of the obese state (24-27). (28). It has been demonstrated that NLRP3 is also important in the airways, as in an allergic mouse model (29) the absence of NLRP3 and IL-1β reduced the expression of IL-5, IL-13 and IL-3. Furthermore, in experimental models of severe, steroid resistant asthma, we have shown that NLRP3 and IL-1β responses are increased; that therapeutically targeting NLRP3 reduces IL-1β production, neutrophilic inflammation and airways hyper-responsiveness; and that administration of IL-1β induces severe, steroid-resistant neutrophilic asthma (30). We also observed an association between NLRP3/ IL-1β and neutrophil numbers and disease severity in asthmatic patients (30). Here we report increased NLRP3 gene expression and increased protein levels of IL-1β in obese asthmatic airways, complementing a previous report of increased sputum IL-1β following ozone exposure in obese subjects (31). Interestingly, in females only, IL-1β gene expression correlated with NLRP3 and TLR4 gene expression and %sputum neutrophils, which suggests that our previous report of increased sputum %neutrophils in female obese asthmatics (10) may be a result of gender specific NLRP3 activation. This may be clinically important, as IL-1β expression was inversely correlated with lung function.

The upregulation of IL-5 expression in obese asthma and the correlation between IL-5 and NLRP3 suggests that the NLRP3 inflammasome may enhance Th2 cytokine production. Airway eosinophilia is a hallmark of asthma and IL-5 is the main cytokine associated with eosinophil development, mobilization, activation, redistribution and survival (32). However, in the current study, sputum %eosinophils were not increased in obese asthmatics, which agrees with previous research from our group and others (11, 33, 34). A recent study in obese mice reported lower eosinophil counts in airway lining fluid, despite higher IL-5 and eotaxin levels and higher eosinophil counts in the bone marrow and surrounding lung tissue (35). Another recent clinical study showed that airway sub-mucosal eosinophil numbers, but not sputum eosinophils, were higher in obese subjects (34). Collectively, these results suggest that obesity promotes eosinophil trafficking from bone marrow to the lung, but inhibits transit into the airways. They also suggest that other factors, possibly eotaxins, may be involved in eosinophil responses in obese asthma. The clinical relevance of eosinophils in the mucosa versus lung tissue requires investigation.

Using acute meal challenges, we examined the postprandial effects of the macronutrients typically found in obesogenic diets; SFA, n-6 PUFA and simple carbohydrates. We found that both types of high fat meals led to increases in circulating blood neutrophils and leukocytes, which agrees with previous reports of increased blood neutrophils following a high SFA meal (36, 37) or high fat mixed meal (38). Our observation that dietary fat is more pro-inflammatory than carbohydrate, is also supported by previous acute feeding studies which have shown that whilst both fat and carbohydrate increase circulating neutrophils (36), NF-κB activity and increased TNF-α and IL-1β expression in mononuclear cells (39), dietary fat induces the greatest effect (36, 39). Increased migration of leukocytes occurs following a high fat meal, as cells exposed to excess free FAs are activated and subsequently release chemokines such as CXCL8 and adhesion molecules such as CD11a (37). Indeed,

neutrophils isolated from peripheral blood following a SFA meal display increased chemotaxis towards complement factor 5a (C5a), CXCL8 and *N*-formyl-methionyl-leucyl-phenylalanine (37).

We observed an increase in airway inflammation (sputum %neutrophils) following the saturated fat meal, in non-obese subjects only. We have previously reported that neutrophilic airway inflammation is increased following a high fat, high energy mixed meal, which was high in saturated fat, but also carbohydrates and n-6 PUFAs (14). The current study has allowed us to determine which macronutrients activate inflammatory pathways in asthmatic airways. Here we have demonstrated that only saturated fat increases sputum %neutrophils, which is in agreement with the positive correlation that we previously reported between the change in plasma SFA and the change in sputum %neutrophils following the high fat mixed meal (14). This also provides a potential explanation for previous observations that saturated fat intake is associated with increased asthma risk (40, 41).

Interestingly, there were no changes in airway inflammation in obese asthmatics, likely due to desensitization of obese asthmatics due to chronic exposure to high circulating FA levels. Indeed, we have shown that obese asthmatics have chronically elevated circulating FA levels, likely to result from a combination of both excessive fat intake *and* metabolic abnormalities, which impair the homeostatic mechanisms responsible for cellular uptake and storage of nutrients and maintenance of optimal circulating fatty acid levels (42). They also had higher basal expression of the NLRP3 inflammasome and increased IL-1β release. Hence, it appears that obese asthmatics do not experience further postprandial upregulation of inflammation. Desensitization of inflammatory responses in asthmatics following chronic exposure to external stimuli, such as endotoxin, has been previously reported (43, 44).

In the non-obese asthmatics following the SFA intervention, there were 9 differentially expressed genes. These included NLRP3, TLR4, IL-1 receptor agonist (IL-1RN) and IL-1 receptor accessory protein (IL-1RAP), which also suggest NLRP3 inflammasome activation. TLR4 was upregulated, demonstrating the first stage of inflammasome activation, as SFAs bind to TLR4, inducing NF-κB activity and inflammatory cytokine production (14, 45). This concurs with our previous postprandial study that showed increased gene expression of TLR4 following a high fat, high energy mixed meal (14). Here we also show upregulation of TLR6 gene expression, which others have shown to form a heterodimer with TLR2, which can be triggered by SFA to activate an NF-κB-driven inflammatory cascade (46). Further, the IL-1 pathway was modified by the SFA meal in non-obese asthmatics, with transcripts of both IL-1RN and IL-1RAP being upregulated. IL-RN is the gene that encodes the IL-1 receptor antagonist (IL-1RA) protein, which functions as an anti-inflammatory cytokine by inhibiting the activities of IL-1α and IL-1β through interaction with the IL-1 receptor (IL-1R) (47). IL-1RAP enhances IL-1 activity, being a necessary component of the IL-1R complex that initiates signalling events resulting in activation of IL-1-responsive genes. As described above, our previous studies have shown the importance of IL-1 pathway in inducing steroid-insensitive neutrophilic responses in mouse models (30) and predicting future exacerbations of airways disease (48).

Our studies have several limitations. The studies presented in this paper examine NLRP3 inflammasome activation in asthmatic airways, in relation to obesity and saturated fat intake. As airway NLRP3 inflammasome activity increases in non-asthmatics with other exposures, such as ozone (31), we cannot exclude the possibility that obesity and saturated fat would also activate the NLRP3 inflammasome in non-asthmatic airways. The clinical implications of such a finding are unknown and this is an interesting area for further investigation. The cross sectional analysis cannot establish cause and effect, so our observations need to be further explored in longitudinal studies. Furthermore, the effect of co-morbidities such as obstructive sleep apnoea, on chronic and postprandial inflammation, are poorly understood and should be considered in future research. The acute meal study gene expression analysis had a small sample size, as paired sputum samples could not be collected from some individuals. This is difficult to address, as the study design required two sputum inductions within 4 hours, which reduces the likelihood of obtaining a sufficient volume of mucus plugs on the second collection. Nonetheless, we had adequate samples to identify changes in airway cell numbers, which allowed us to focus our gene analysis. Another limitation of this study is the absence of data on usual diets. This should be included in future studies to explore the effect of chronic nutrient intake on postprandial responses. Finally, our studies examined gene expression in sputum samples, which are a heterogeneous mixture of inflammatory cells. We have addressed this limitation by using *ex vitro* experiments to confirm specific cell types that are activated by SFAs. Our *ex vivo* experiments also provide evidence of the tightly controlled two-stage process by which inflammasome activation occurs. In both neutrophils and monocytes, we found that a combination of both free saturated fatty acid (palmitic acid) and LPS/TNFα were required to induce IL-1β release.

Our analyses provide insight into the nature of airway inflammation in asthmatics with a high BMI and in the postprandial phase. We have identified the NLRP3 inflammasome as being differentially regulated in obese asthmatics and highlighted several targets, including NOD1 and NLRP3, which could be furthered explored to develop improved therapies for managing obese asthma. Our study has also confirmed and extended our previous observation that a high fat, high energy meal induces neutrophilic airway inflammation. We have shown that SFAs are the responsible macronutrient and the effect is confined to non-obese asthmatics. We have also identified several genes involved in SFA-induced neutrophilic airway inflammation, including TLR4/6 and NLRP3, which provide possible therapeutic targets for immunomodulation. This study provides translational outcomes, suggesting that both reversal of obesity in asthma and restriction of dietary saturated fat intake in non-obese asthmatics warrant further investigation as anti-inflammatory strategies.

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Table 1: Nutrient composition of the isocaloric study meals: SFA, n-6 PUFA and carbohydrate meals. Subjects consumed the following meals after an overnight fast, in addition to 200g mashed potato provided at time 0 hours (720 kJ, 0.2g fat, 40g carbohydrate).

	Carbohydrate Meal	n-6 PUFA Meal	SFA Meal
Energy (kJ)	1850	1740	1840
Fat (g)	<1	47	48
Fat (%energy)	<1	100	>98
Saturated fatty acids (g)	0	4	34
Saturated fatty acids (%energy)	0	9	70
n-6 PUFA (g)	0	33	2
n-6 PUFA (%energy)	0	72	4
Carbohydrate (g)	100	0	<1
Carbohydrate (%energy)	>99	0	<1
Protein (g)	0	0	<1
Protein (%energy)	0	0	<1

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Table 2. Clinical characteristics, sputum cell counts and plasma fatty acid concentrations of obese and non-obese subjects in Study 1

	Non-obese (n=51)	Obese (n=76)	*p*-Value*
Clinical characteristics			
Age (years)	48 (16)	52 (14)	0.231
Gender (M|F)	19|32	33|43	0.488
BMI (kg/m^2)[a]	26.1±2.7	35.6±4.8	NA
Atopy, n(%)	30 (70)	33 (73)	0.711
FEV1%predicted[a]	79.4±19.6	81.3± 18.8	0.591
FVC%predicted[a]	94.1±16.8	88.9±16.1	0.080
FEV1/FVC%[a]	67.7±10.9	72.8±9.6	0.006
ICS dose (ug/day)[c]	500 (0-1000)	500 (200-1000)	0.355
SABA use, n(%)	45 (88)	67 (88)	0.989
LABA use, n(%)	31 (61)	55 (72)	0.173
ACQ (units)	1.0 (0.4-1.6)	1.0 (0.6-1.4)	0.668
eNO (ppb)	18 (10-38)	20 (13-31)	0.568
Sputum cell counts[b]			
Total cell count (10^6/mL)	3.1 (2.4-5.3)	2.1 (0.8-5.0)	0.013
Neutrophils%	38.0 (24.0-54.8)	49.5 (24.5-64.3)	0.305
Neutrophils (10^4/mL)	128.0 (53.0-301.0)	94.7 (24.4-259.2)	0.177
Eosinophils%	1.5 (0.7-6.4)	2.0 (0.5-4.8)	0.932
Eosinophils (10^4/mL)	6.7 (2.1-18.3)	5.1 (0.9-13.4)	0.287
Macrophages%	52.8 (35.8-65.5)	43.5 (29.3-64.8)	0.341
Macrophages (10^4/mL)	177.5 (114.1-235.3)	98.3 (34.1-202.9)	0.001
Plasma fatty acids (mg/L)[a]			
C10:0	1.1±0.2	1.7±1.1	0.208
C14:0	37.2±3.2	39.3±3.5	0.374
C16:0	710.0±39.4	807.8±45.5	0.042
C18:0	214.9±11.5	245.3±12.7	0.014
C18:2 n-6	877.9±30.8	940.3±41.0	0.274
C20:4 n-6	313.1±14.8	357.1±14.9	0.022
SFA	1017.0±54.9	1154.1±63.3	0.035
MUFA	887.1±55.1	1042.4±58.8	0.006
PUFA	1571.2±49.9	1701.4±62.8	0.107
Total FA	3475.3±153.7	3897.9±175.9	0.029

ACQ, Asthma Control Questionnaire; BMI, body mass index; C10:0, capric acid; C14:0, myristic acid; C16:0, palmitic acid; C18:0, stearic acid; C18:2 n-6, linoleic acid; C20:4 n-6, arachidonic acid; eNO, exhaled nitric oxide; FEV1%, forced expiratory volume in 1 second as percentage of predicted value; FVC%, forced vital capacity as percentage of predicted value; ICS, inhaled corticosteroids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; [a]Mean±SD; [b]Median (Quartile 1–3); [c]beclomethasone equivalents; *Parametric data were analyzed using the unpaired t-test and non-parametric data were analyzed using the Mann Whitney U test.

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Table 3. Sputum cell genes differentially expressed (>1.5 fold change) in obese (n=11) versus non-obese (n=14) asthmatics in Study 1

Gene Symbol	Gene Name	Fold change	*p* value (Obese vs Non-obese)
IL5	interleukin 5	5.4	0.003
NOD1	nucleotide binding oligomerization domain containing 1	1.8	0.015
TREM2	Triggering receptor expressed on myeloid cells 2	-2.4	0.023
GNGT1	G protein subunit gamma transducin 1	3.1	0.024
FASLG	Fas ligand	4.2	0.025
IRF7	Interferon regulatory factor 7	1.5	0.031
NLRP3	NLR family, pyrin domain containing 3	2.5	0.035
IL9	interleukin 9	3.1	0.041
IL12B	interleukin 12B	4.0	0.046
TBXA2R	thromboxane A2 receptor	1.8	0.046
C1R	complement component 1, r subcomponent	3.0	0.048
TGFBR1	transforming growth factor beta receptor 1	-1.5	0.048
TGFB2	transforming growth factor beta 2	2.2	0.050

Data were assessed using unpaired t-tests with the Benjamini Hochberg adjustment for multiple comparisons.

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Table 4. Characteristics of asthmatic subjects who completed the acute meal challenge study in Study 2 (Visit 1, time=0 hours)

	All samples	Non-obese	Obese	*p* value (Obese vs Non-obese)[a]
N	23	12	11	NA
Age (years)	46.0(16.5)	41.2(16.8)	51.3(15.1)	0.145
Gender (F|M)	16|7	7|5	9|2	0.221
BMI, mean±SD	30.3±4.7	26.7±2.0	34.3±3.5	NA
Atopy, n (%)	13 (62)	8 (67)	5 (56)	0.060
FEV1%, mean±SD	80.9±19.6	79.6± 22.9	82.5±16.3	0.730
FVC%, mean±SD	87.9±14.9	88.3±15.7	87.4±14.7	0.887
FEV1/FVC (%), mean±SD	73.4±11.8	72.4±14.4	74.6±8.7	0.665
ACQ (units), median (Q1-Q3)	0,7 (0.4-1.1)	0.8 (0.3-1.0)	0.7 (0.6-0.7)	0.337
ICS use (υg/day)[b], median (Q1-Q3)	1000 (750-2000)	625 (0-1000)	1000 (0-1750)	0.459
Sputum cell counts. median (Q1-Q3)				
Total cell count (x10^6/mL)	3.7 (1.7-5.5)	2.5 (1.4-6.3)	4.7 (2.3-5.6)	0.472
Neutrophils%	24.8 (12.7-48.8)	23.5 (14.0-43.3)	25.3 (10.4-55.2)	0.762
Eosinophils%	0.8 (0.5-2.4)	0.8 (0.4-2.7)	1.3 (0.4-2.6)	0.593
Macrophages%	64.0 (44.1-78.4)	58.3 (48.6-71.4)	70.0 (39.8-80.7)	0.650
Blood cell counts (x10^9/L). Median (Q1-Q3)				
Total white cells	5.9 (5.0-6.8)	5.2 (4.4-7.0)	6.0 (5.5-6.7)	0.308
Neutrophils	3.4 (2.8-3.8)	3.3 (2.3-3.6)	3.4 (3.2-4.2)	0.411
Lymphocytes	1,9 (1.5-2.1)	1.6 (1.3-2.0)	1.9 (1.5-2.2)	0.339
Monocytes	0.4 (0.3-0.6)	0.4 (0.3-0.6)	0.4 (0.4-0.6)	0.787
Eosinophils	0.1 (0.1-0.3)	0.2 (0.1-0.3_	0.1 (0.1-0.3)	0.697

BMI, body mass index; FEV1%, forced expiratory volume in 1 second as percentage of predicted value; FVC%, forced vital capacity as percentage of predicted value. [a]Parametric data were analyzed using the unpaired t-test and non-parametric data were analyzed using the Mann Whitney U test. [b]beclomethasone equivalents. Kruskal Wallis testing confirmed that there were no differences in the baseline (time=0 hours) cell counts for each of the study meals.

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Table 5. White blood cell and sputum supernatant counts of subjects at baseline and 4 hours after each isocaloric meal in Study 2

Blood ($x10^9$/L)	Carbohydrate (n=21)			n-6 PUFA (n=22)			SFA (n=21)			
	0h	4h	p†	0h	4h	p†	0h	4h	p†	p#
Total white cells	5.5(5.0,6.3)	6.2(5.4,7.4)	0.010	5.8(5,6.7)	6.6(6.2,7.5)	0.001	6.2(5.4,6.8)	7.3(6.7,8.4)	0.001	0.034 ₮
Neutrophils	3.2(2.6,3.5)	3.6(3.3,4.5)	0.062	3.2(2.8,3.6)	3.9(3.6,4.4)	0.001	3.9(2.8,4.3)	4.3(3.5,5.6)	0.001	0.036 ₮
Lymphocytes	1.7(1.5,2.1)	1.9(1.6,2.3)	0.001	1.9(1.5,2.1)	2.1(1.7,2.5)	0.001	1.9(1.5,2.2)	2.1(1.7,2.5)	0.002	0.476
Monocytes	0.4(0.3,0.5)	0.5(0.4,0.6)	0.019	0.4(0.3,0.5)	0.4(0.4,0.5)	0.040	0.4(0.3,0.5)	0.5(0.4,0.6)	0.007	0.347
Eosinophils	0.1(0.1,0.3)	0.1(0.1,0.3)	0.620	0.2(0.1,0.3)	0.2(0.1,0.3)	0.119	0.2(0.1,0.3)	0.2(0.1,0.2)	0.565	0.194
Sputum	Carbohydrate (n=20)			n-6 PUFA (n=20)			SFA (n=19)			
	0h	4h	p†	0h	4h	p†	0h	4h	p†	p#
Total cell count ($x10^6$/L)	3.6(2.6,6.4)	2.0(1.2,3.7)	0.003	2.6(1.3,6.0)	2.1(0.6,3.2)	0.012	3.0(1.5,4.6)	2.0(1.4,3.5)	0.382	0.745
Neutrophils%	36.5(16.6,56.4)	47.6(11.8,59.8)	0.266	34.9(20.5,59.8)	28.1(14.8,60.8)	1.000	33.0(11.8,57.5)	44.9(26.8,60.3)	0.140	0.530
Eosinophils%	1.1(0.3,3.1)	1.4(0.3,3.0)	0.849	0.8(0.6,2.3)	1.8(0.4,2.8)	0.029	1.0(0.5,2.8)	1.3(0.5,4.0)	0.077	0.180
Macrophages%	58.1(41.3,77.6)	42.8(31.7,64.0)	0.049	54.3(35.6,71.6)	50.0(31.8,67.3)	0.044	53.3(38,8.8)	50.5(23.5,57.5)	0.056	0.883
Lymphocytes%	0.9(0.0,3.4)	1.0(0.5,2.5)	0.304	1.9(0.5,2.8)	1.5(0.63,5)	0.238	1.3(0.5,4.5)	0.63(0.3,1.8)	0.209	0.208

Data is Median (Quartile 1 – Quartile 3); †comparison of 4hr vs 0hr (analyzed using the Wilcoxon signed rank test); #comparison of change following each meal (analysed using repeated measures ANOVA); ₮P<0.05 for CHO vs SFA (analysed using General Linear Model with atopy as a covariate).

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Table 6. Sputum cell genes differentially expressed (>1.5 fold change) at 4 hours after consumption of a SFA meal by non-obese asthmatics (n=4) in Study 2

Gene Symbol	Gene name	Fold change	*P* value (4hr vs 0 hr)
NLRP3	NLR family, pyrin domain containing 3	2.9	0.003
TLR4	toll-like receptor 4	1.5	0.003
IL1RN	interleukin 1 receptor antagonist	2.7	0.007
PRKCB	protein kinase C beta	2.0	0.009
PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	3.2	0.010
TLR6	toll-like receptor 6	1.5	0.010
CXCL10	chemokine (C-X-C motif) ligand 10	-2.1	0.010
IL1RAP	interleukin 1 receptor accessory protein	2.0	0.025
CSF2	colony stimulating factor 2 (granulocyte-macrophage)	2.4	0.026

Data were analysed using paired t-tests with the Benjamini Hochberg adjustment for multiple comparisons.

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Figure Legends

Figure 1: IL-1β protein concentration in sputum supernatant from non-obese (n=51) versus obese (n=76) asthmatics. Data were analyzed using the Mann Whitney U test.

Figure 2: Associations between BMI, NLRP3 and IL1β levels. a) Sputum cell NLRP3 gene expression and BMI (r=0.585 and p=0.003), b) Sputum supernatant IL1β concentration and BMI (r=0.496 and p=0.012), and c) Sputum cell NLRP3 gene expression and sputum supernatant IL1β concentration (r=0.679, p<0.001). Associations were examined using Spearman's rank correlation coefficient.

Figure 3: a) Changes in white blood cell counts after each isocaloric meal in non-obese and obese subjects, b) Changes in sputum cell counts after each isocaloric meal in non-obese and obese subjects. *p<0.05 for comparison of 4 hours versus baseline (analyzed using the Wilcoxon signed rank test); **p<0.05 for between group comparison (analyzed using repeated measures ANOVA).

Figure 4: Sputum cell genes differentially expressed (>1.5 fold change) at 4 hours after consumption of a SFA meal by non-obese asthmatics, interact in a network consisting of 8 genes. Data were assessed using paired t-tests with the Benjamini Hochberg adjustment for multiple comparisons. Search Tool for the Retrieval of Interacting Genes, STRING v10 was used to investigate pathway interactions between differentially expressed genes using medium confidence scores >0.4. All nodes are coloured indicating they are first shell interactions. Green lines represent interactions determined by text mining, black lines represent interactions that are co-expressed, blue lines are known interactions from curated databases, and purple lines refer to protein homology.

Figure 5: IL-1β release from a-b) neutrophils and c-d) monocytes collected from healthy control subjects, following stimulation with LPS (a,c) and TNFα (b,d) alone and in combination with palmitic acid. *p<0.05 versus controls; **p<0.001 versus controls. Data were analyzed using repeated measures ANOVA.

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Figure 1:

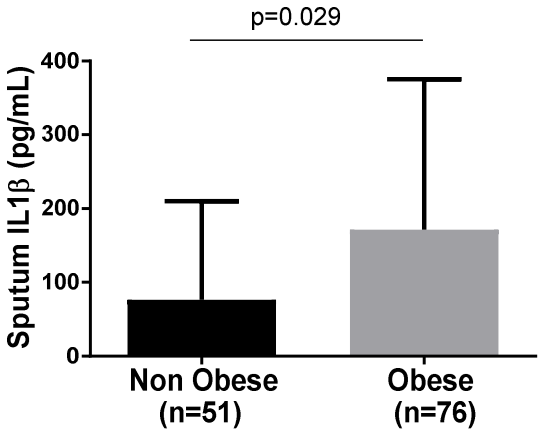

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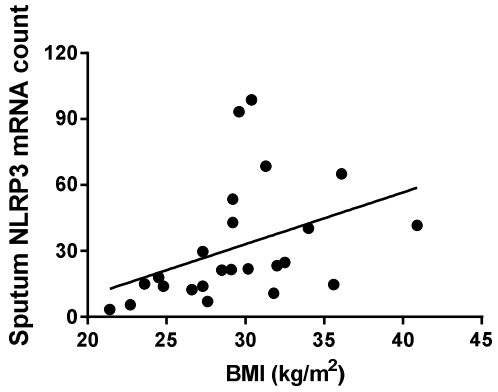
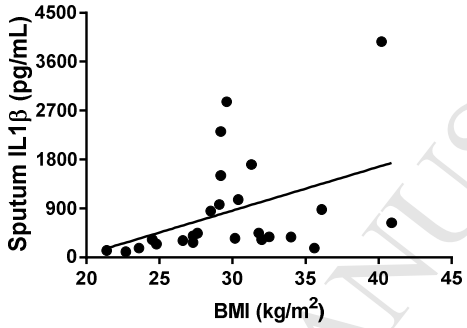
Figure 2:

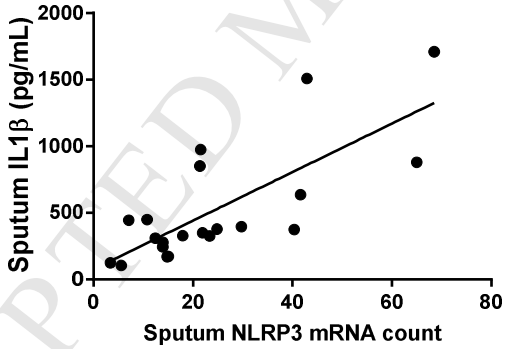
a)



b)

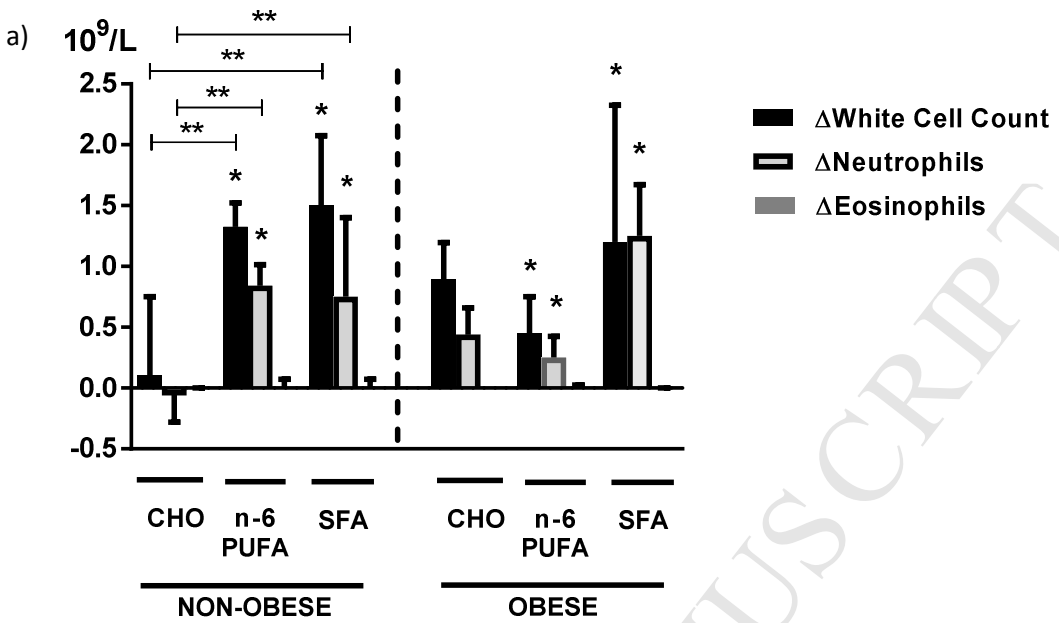


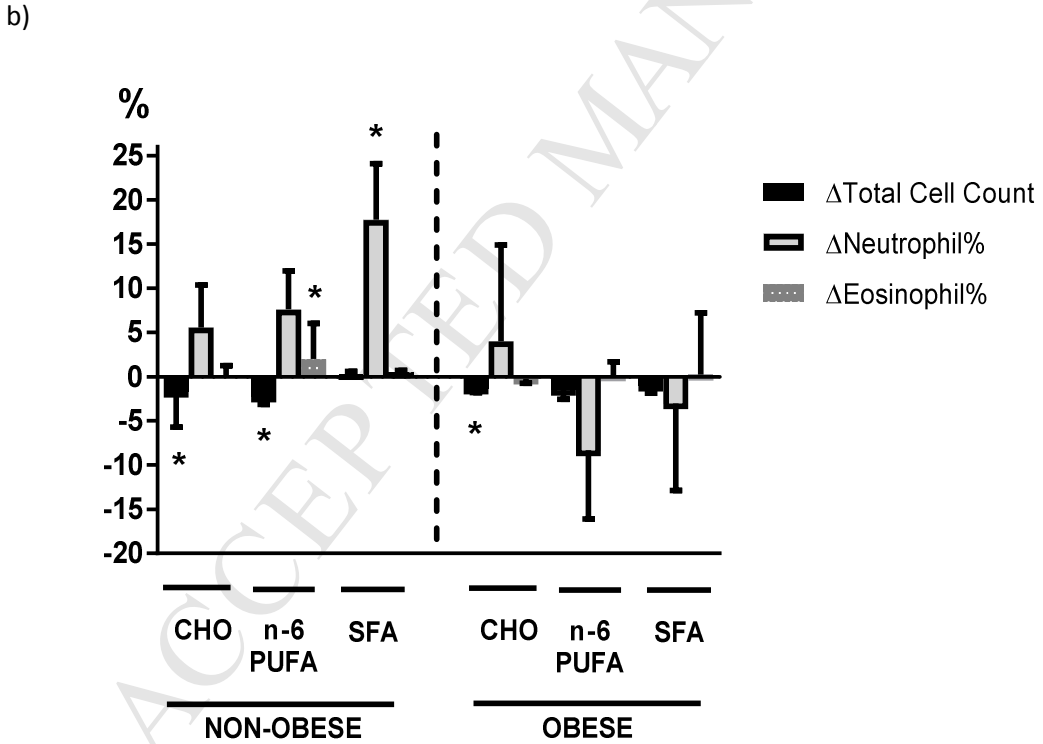
c)



ACCEPTED MANUSCRIPT

Figure 3:





ACCEPTED MANUSCRIPT

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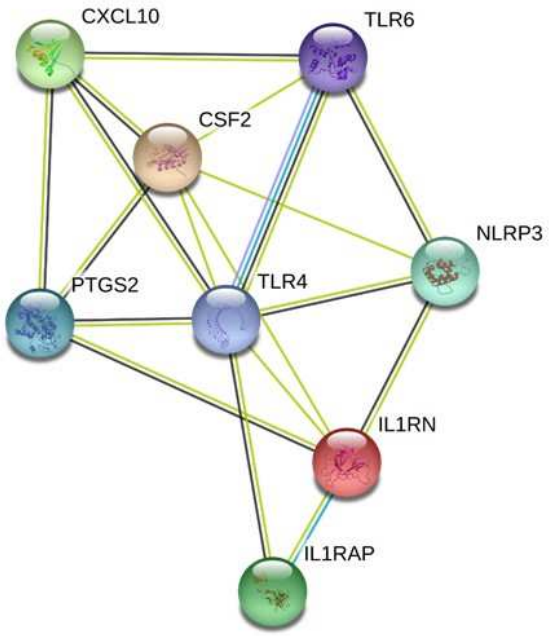
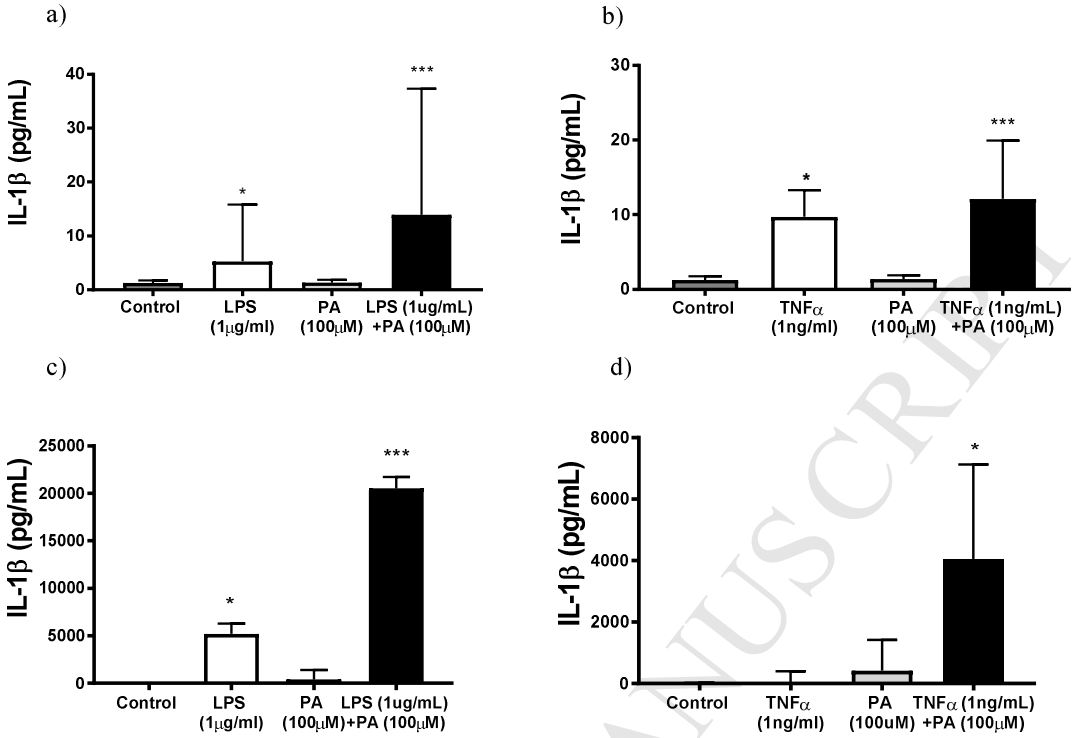


ACCEPTED MANUSCRIPT

Figure 5:



ACCEPTED MANUSCRIPT