

Reduced dietary intake of pro-inflammatory Toll-like receptor stimulants favourably modifies markers of cardiometabolic risk in healthy men

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Keywords

Toll-like receptor, inflammation, atherosclerosis, processed food, LDL-cholesterol

Abbreviations

BLP, bacterial lipoprotein; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostatic model of insulin resistance; hsCRP, high sensitivity C-reactive protein; LDL-C, low-density lipoprotein cholesterol; LPS, lipopolysaccharide (endotoxin); PAMP, pathogen-associated molecular pattern; PBMC, peripheral blood mononuclear cells; TLR, Toll-like receptor; WBC, white blood cell.

Abstract

Background and aims: Because pro-inflammatory stimulants of Toll-like receptor-2 and TLR-4 (pathogen-associated molecular patterns, PAMPs), are abundant in some processed foods, we explored the effects of diets enriched or depleted in these molecules on markers of cardiometabolic risk in man.

Methods and results: Adherence to a low PAMP diet for 7 days reduced LDL-cholesterol (-0.69 mM, $P=0.024$) and abdominal circumference (-1.6 cm, $P=0.001$) in 11 habitual consumers of high PAMP foodstuffs, and these markers, together with leukocyte counts (+14%, $P=0.017$) increased significantly after 4 days consuming predominantly high PAMP foods. Change in LDL-cholesterol and leukocyte counts correlated well with change in frequency of intake of high PAMP foodstuffs per individual ($r=0.540$, $P=0.0095$ and $r=0.6551$, $P=0.0009$, respectively). In an independent group of 13 healthy men, leukocyte counts and expression of the activation marker CD11b on granulocytes and monocytes were significantly reduced after a fresh onion meal ($P<0.05$), but these effects were reversed by a high PAMP equivalent meal.

Conclusions: A low PAMP diet is associated with reduced levels of several cardiometabolic risk factors, while a high PAMP diet reverses these effects. These findings suggest a novel potential mechanistic explanation for the observed association between processed food consumption and risk of cardiometabolic diseases. The study is registered at clinicaltrials.org (reference NCT02430064).

Introduction

Evidence is accumulating to suggest that chronic metabolic diseases, including type II diabetes and atherosclerosis, may be promoted by low-grade inflammation induced by specific dietary patterns [1-3]. For example, observational studies have reported that the consumption of processed meats, relative to unprocessed meats, is associated with elevated C-reactive protein (CRP) levels [4], risk of type II diabetes [4,5] and risk of cardiovascular disease [6,7]. However, the specific components of these diets which are responsible for promoting inflammatory signalling, or metabolic risk, remain to be clearly defined.

Recent evidence suggests that conserved molecules of microbial origin termed pathogen-associated molecular patterns (PAMPs) may play a key role in this relationship [8,9]. In particular, parenteral administration of PAMPs which stimulate the innate immune receptors Toll-like receptor (TLR)-2 or TLR4 (bacterial lipopeptides, BLP, and lipopolysaccharides, LPS, respectively), promotes inflammation, insulin resistance, impaired reverse cholesterol transport and atherosclerosis in murine models [8-10]. Circulating endotoxin levels also correlate positively with risk of atherosclerosis and type II diabetes in man [11].

Because the human large intestine contains a very large number of bacteria (~100 trillion), and a high PAMP content [12], it has been widely assumed that food-borne TLR-stimulants are unlikely to be significant contributors to systemic inflammatory tone. However, we found recently that of the four major phyla that dominate the human intestinal microbiota (Bacteroidetes, Actinobacteria, Firmicutes and Proteobacteria), only key marker species of the numerically minor (~0.1%) Proteobacteria group secreted large quantities of soluble stimulants of TLR2 and TLR4 [12]. Accordingly the soluble PAMP content of the human faecal microbiota was much lower than expected [12].

By comparison, we found that a variety of commonly consumed foodstuffs associated with the Western diet, particularly processed foods containing minced meats or chopped onion

stored at refrigeration temperature, frequently contained bacterial TLR-stimulants at concentrations that were several orders of magnitude higher than those measured in the healthy murine small intestine, which normally has a very low PAMP content but is the major site of endotoxin absorption [12-14]. The present study was therefore conducted to explore the hypothesis that dietary TLR-stimulants may modify systemic markers of inflammation and cardiometabolic risk in human volunteers *in vivo*.

Materials and methods

Recruitment and subjects

Two studies were conducted, one chronic sequential dietary intervention sampling at three timepoints over 12 days and one acute, single blinded, cross-over study with 2 interventions each sampled on three timepoints over 24 h (see Supplemental Figure 1 for a graphical depiction of the study protocols). For the chronic study, 15 healthy male volunteers (age 37.5 ± 10.0 years) were recruited, of whom 4 reported onset of mild infections during the trial and were excluded from further study. For the acute study, 13 healthy male volunteers (age 27.8 ± 10.7 years) were recruited and all completed the study. Baseline physical characteristics of each cohort are summarised in Supplemental Table 1. For both studies, inclusion criteria were healthy men between the ages of 18 and 65. Exclusion criteria included evidence of any current inflammatory condition, infection or vaccination within two weeks prior to the study and use of medications. All subjects gave informed consent, and ethical approval for the study (reference NCT02430064 at clinicaltrials.org) was granted by the University of Leicester College of Medicine Ethics Committee.

Study design and dietary interventions

For the chronic study, volunteers were asked to avoid specific types of food we found in recent studies to be at relatively high risk of containing high levels of PAMPs [14,15], and to consume any quantity of fresh produce, including any form of meat, fish or vegetables that had not been minced or processed unless immediately before consumption, for a run-in period of 7 days. There were no restrictions on salt, sugar or non-alcoholic beverages. Fasting blood samples were collected on days 0 and 7 of this run-in period. Then, over the next 4 days, subjects consumed a set lunch and evening meal provided to them, each chosen on the basis of high PAMP content from prior screens (Figure S2, Table S2). Subjects were asked to maintain a quantitative diet diary and to avoid excessive alcohol consumption for the duration of the study. A more detailed description of dietary advice, nutrient and PAMP content of provided food items, **methods for assessing dietary intake and power calculations** is provided in the Supplemental Methods.

The acute study was of single-blinded, crossover design. 13 healthy male volunteers fasted overnight before giving blood then ingesting either a low PAMP (control) or high PAMP onion-based breakfast on separate occasions with at least two weeks washout between visits. Subjects provided a postprandial blood sample at 3 h and a second fasted sample at 24 h. The low and high PAMP meals differed only in the PAMP content of the onion used to prepare each meal, and were otherwise nutritionally identical. Detailed methods for test meal preparation and composition, biochemical measurements and nutritional analyses are provided in the Supplemental Methods section.

Statistics

For the chronic study, responses were compared **using linear mixed models with Sidak's post-test**. For the acute study, linear mixed models were used with meal and time as within **subject factors**. Associations between cytokine production and TLR-stimulant contents of

foods were tested by Spearman correlation. Data were analysed using Graphpad Prism 6 and IBM SPSS 22, and are presented as mean \pm SE unless otherwise indicated. Statistical significance was assumed at $P < 0.05$.

Results

PAMP content of test meals and diets

To identify foods at high risk of inducing inflammatory signalling for use in the study, a panel of 23 potential test meals purchased from local supermarkets was screened to identify inducers of the inflammatory cytokines IL-6, IL-1 β and TNF- α by human whole blood. Production of each of these cytokines correlated strongly with food content of soluble TLR4-stimulants, as measured using HEK-293-TLR4/MD2 transfectants (all $r > 0.65$, $P < 0.001$, Figure S2), and also TLR2-stimulants (all $r > 0.48$, $P < 0.05$). As expected, numbers of viable aerobic mesophile bacteria increased with time during storage at refrigeration temperature in four representative pre-prepared meals (lasagne and bolognese from two suppliers, Figure S3). Notably, concentrations of TLR2 and TLR4 stimulants in two of the tested meals were already relatively high long before the advertised 'best before' date, and while bacterial numbers were still low, raising the possibility that the majority of TLR-stimulants in these products entered the supply chain prior to assembly and pre-cooking of the ingredients (Figure S4).

Recall questionnaires revealed that the volunteers recruited to the chronic study were habitual consumers of foodstuffs identified in earlier studies to be at relatively high risk of containing high levels of PAMPs (i.e. processed meats, pre-prepared meals, cheese and chocolate, which commonly contain > 100 ng/g LPS or BLP [14-16]), consuming on average 1.4 servings of these items per day. Diet diaries confirmed that the study interventions significantly reduced the number of servings of high PAMP foods to 0.5 per day ($P = 0.002$)

during the low PAMP run-in week and these increased to 4.8 servings per day during the high PAMP arm ($P<0.001$, Table 1). The meals provided to volunteers during the high PAMP period contained on average 73.5 μg Pam₃CSK₄-equivalent TLR2-stimulants, and 3,109 μg *E. coli* LPS-equivalent TLR4-stimulants per day. Estimated typical daily nutrient intakes based on the diet diaries revealed similar levels of consumption of total fat, carbohydrate, sugar, fibre, protein and salt, although saturated fat intake was estimated to be ~75% higher during the high PAMP arm of the study (Table S3).

Effects of chronic intake of high PAMP meals on systemic markers of inflammation and metabolism

Total leukocyte count was reduced by the low PAMP dietary intervention (-12%, $P=0.024$), but increased significantly from this baseline after 4 days of high PAMP feeding (+14%, $P=0.017$, Figure 1). This was driven by significant increases in numbers of granulocytes (+12%, $P=0.028$) and lymphocytes (+17%, $P=0.019$). At the individual level, there was a strong correlation between achieved change in daily intake of high PAMP foods and change in leukocyte counts ($r=0.6551$, $P=0.0009$). However, there were no significant changes to high sensitivity CRP (Figure 1). Unexpectedly, significant reductions in weight (-0.7 kg, $P=0.028$) and abdominal circumference (-1.6 cm, $P=0.001$) were also recorded after the 7 day low PAMP diet (Figure 2). Waist measurements, but not weight, increased significantly after the high PAMP intervention (1.2 cm, $P=0.025$), paralleling a similar fall and restoration of serum leptin concentrations. Although the dietary interventions had no significant effect on markers of insulin sensitivity (insulin, glucose and HOMA-IR), LDL-C was significantly reduced by the low PAMP diet (-0.69 mM, $P=0.024$) and this was again reversed by the high PAMP intervention (Figure 3). Accordingly, at the individual level, change in LDL-C correlated well with achieved change in frequency of high PAMP food

consumption ($r=0.540$, $P=0.0095$). Blood pressure, serum endotoxin and endotoxin neutralisation capacity values, as measured using the limulus assay, were not significantly altered by diet (Figure S5).

Acute effects of a single high PAMP meal on systemic markers of inflammation and metabolism

As it was likely that daily intake of saturated fat and food additives, such as surfactants and preservatives, differed substantially between the two arms of the chronic study, we could not rule out a potential role for these agents in the regulation of inflammatory status from the chronic study alone. A second study was therefore designed based on two meals that differed in their content of bacterial products, but which were otherwise nutritionally identical. To achieve this, we made use of the prior observations that while TLR-stimulants are undetectable in freshly chopped onion, γ -proteobacteria (which are a major source of soluble TLR2/4-stimulants [15,16]) grow rapidly in chopped onion stored at refrigeration temperature [16]. Repeated testing of ready-chopped onion from a particular vendor revealed reproducibly high content of PAMPs at the ‘best before’ date. Test meals for the acute study were therefore prepared using either 200 g freshly chopped onion (low PAMP meal), or 200 g ready-chopped onion (high PAMP meal). TLR-transfectant based bioassays confirmed that the two meals differed significantly in their content of TLR2- and TLR4-stimulants (21.0 ± 5.5 vs 746 ± 324 μg , $P=0.0286$ and 14.6 ± 5.3 vs 379 ± 153 μg , $P=0.0286$, respectively). Nutritional contents of the complete meals were otherwise identical (Table S4).

Apart from a significant increase in PBMC CCL-2 mRNA postprandially (38%, $P=0.046$, Figure S6), we did not observe significant increases in inflammatory markers within 24 h after the high PAMP onion meal. Unexpectedly, however, the fresh onion meal caused significant reductions in several leukocyte markers of inflammatory status. For example,

leukocyte counts were significantly reduced 24 h after the low PAMP onion meal ($P=0.013$, Figure S7), and flow cytometry revealed that surface expression of CD11b, which is a sensitive marker of leukocyte activation, was significantly reduced on both monocytes and granulocytes 3 h after the fresh onion meal ($P<0.05$, Figure 4). However, these effects were not observed after the high PAMP meal. Accordingly, monocyte counts tended to fall after the low PAMP meal, but rose after the high PAMP meal ($P=0.041$ for meal*time interaction). Neither meal type induced significant changes to hsCRP, the percentage of CD16⁺CD14^{lo} monocytes or PBMC IL-6, TNF- α or IL-1 β mRNA. Plasma glucose, insulin, lipids, endotoxin, and physical parameters also were not significantly modified by meal type (Figures S8-S10, Table S5).

Discussion

Inflammatory signalling is thought to underpin the development of both atherosclerosis and type II diabetes, yet the responsible stimuli remain poorly defined [1]. Dietary factors are considered to be plausible candidates, since inflammatory markers are elevated among consumers of the Western dietary pattern compared to those consuming predominantly a prudent or Mediterranean dietary pattern [2,3]. However, it remains unclear which components of these diets modify systemic inflammatory tone [17]. We showed previously that many commonly consumed processed foods, but not fresh, unprocessed meats, fruits or vegetables, promote inflammatory cytokine production by human monocytes *in vitro* [14]. These effects were found to be dependent on the release of lipopeptides and lipopolysaccharides by common food-spoilage bacteria which accumulate in foods containing minced meat or finely chopped vegetables when stored at refrigeration temperature for extended periods of time [15,16]. To examine the potential relevance of these findings *in vivo*, we here explored the effects of dietary exposure to foods containing high levels of

lipopeptides and lipopolysaccharides on systemic markers of cardiometabolic risk in healthy human volunteers.

We found that adherence to a high PAMP diet for as little as 4 days caused significant increases in total leukocyte, granulocyte and lymphocyte counts. Notably, these markers are considered to be not only very sensitive, if non-specific, indicators of low-grade inflammation, but also independent risk factors for both type II diabetes and atherosclerosis [18,19]. For example, in cohorts similar to those examined here (i.e. healthy young men), a white blood cell count (WBC) increment of 1,000 cells/mm³ was associated with a 17.4% increase in risk of incident angiographically confirmed CAD, and a 7.6% increase in risk of incident type II diabetes over 7.5 years, after adjustment for other risk factors [18,19]. By way of comparison, the high PAMP diet increased WBC by 630 cells/mm³. Interestingly, the observed changes to WBC count were not paralleled by significant effects of diet on CRP. However, these results are consistent with the observation that infusion of very low-dose (0.3 ng/kg) LPS induces readily detectable increases in leukocyte numbers in human volunteers, even when the increase in inflammatory cytokine production is difficult to detect [20].

The second major finding from the chronic study was the significant lowering of LDL-C by the low PAMP diet, and its rapid reversal by the high PAMP diet. The magnitude of the change (18%) is similar to that observed in volunteers randomised to a combined Mediterranean diet with plant sterol supplement intervention for 4 weeks [21], but greater than reported in trials of individual cholesterol lowering foods, such as psyllium, plant sterols/stanols, soy protein or almonds (which typically reduce LDL-C by ~4-13% [22]). Interestingly, volunteers were not asked to adopt components of a Mediterranean diet, or to increase their consumption of cholesterol lowering foods during the low PAMP arm of the study, and indeed no evidence of their adoption was present in the diet diaries. This suggests that a PAMP exclusion diet may represent a novel potential means of LDL-C lowering.

Although the mechanisms connecting dietary PAMP intake with LDL-C remain to be established, it is interesting to note that in animal models, very low dose (but not high dose) endotoxaemia results in a marked increase in VLDL production [23]. We also note that although daily intake of saturated fat was considerably higher during the high PAMP week, this is unlikely to explain the change in LDL-C, since low saturated fat diets have been reported to reduce LDL-C by only ~3% within 6 months [24]. The difference in saturated fat intake is also not likely to explain the effects on inflammatory markers, since saturated fatty acids were shown not to stimulate TLR2 or TLR4 or otherwise induce classical inflammatory signalling cascades *in vitro* [25].

Previous interventional studies have shown that several plant-based foods, such as grapes or olive oil, promote systemic anti-inflammatory effects in man [26]. To our knowledge, the discovery that fresh onion also induces anti-inflammatory effects *in vivo*, particularly with respect to leukocyte numbers and CD11b expression, has not been reported previously. Nevertheless, these findings are supported by a recent report that onions contain molecules (shown to be quercetin and quercetin-4'-O- β -glucoside) which inhibit TLR2- and TLR4-signalling *in vitro* [27]. Quercetin was also shown to inhibit LPS-induced CD11b activation in human neutrophils *in vitro* [28]. The notion that processed onions promote inflammation may therefore appear counter-intuitive. However, although the bacterial content of fresh onion is very low, it has been shown to increase by a factor of ~30 immediately after factory slicing [29], and the resulting increase in surface area then allows Gram-negative bacteria to reach high levels ($\sim 10^8$ /g) after a short period of storage at refrigeration temperature [16].

If the absorption of PAMPs from dietary sources modifies cardiometabolic risk factors, as suggested by the present findings, this offers a novel potential explanation for the observation that intake of processed meat (e.g. products containing minced meat) confers an increased risk of type II diabetes and cardiovascular disease compared to intake of equivalent quantities

of unprocessed meat [4-7]. Indeed, increased processed meat intake was shown to be associated with a significant increase in risk of diabetes [4,5], elevated CRP [4] and cardiovascular disease [6,7]. By contrast, the Mediterranean dietary pattern is defined in part by relatively low intake of pre-packaged 'ready meals' and processed meats, and is likely a low PAMP diet for these reasons.

The study has some limitations that should be acknowledged. First, because subjects were already habitual consumers of high PAMP foods, the control diet was administered first in order to bring inflammatory markers to a uniform basal level before the high PAMP intervention. Further studies using randomised order may shed more light on the observed effects. Second, the chronic study was not designed to investigate caloric intake or satiety and further studies will be required to address the mechanisms of weight change we observed. Third, the direct measurement of PAMP absorption from the intestinal lumen to the circulation remains very challenging; partly because the half-life of endotoxin, at least as detectable by the limulus assay, in the circulation is ~5 minutes [30], and partly because TLR2/4-stimulants in plasma are below the limit of detection by HEK-293 transfectants. Alternative strategies will therefore need to be developed before the translocation component of the hypothesis can be formally addressed. Finally, the cohorts examined here comprised healthy, young men, while the pathways examined may be more apparent in subjects with impaired intestinal barrier function, such as those with type-II diabetes, non-alcoholic fatty liver disease or obesity.

In conclusion, we present evidence that a low PAMP diet exerts anti-inflammatory effects on leukocyte markers of activation and reduces LDL-C, while a high PAMP diet reverses these effects. These findings suggest that both the pro- and anti-inflammatory potential of foodstuffs may need to be considered in future studies of the effects of dietary constituents on

270 mechanisms of disease. Further studies are merited to determine if low-PAMP diets may also
271 modify inflammatory status, LDL-C, weight or risk of disease in relevant patient groups.

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Table 1: Frequency of consumption of food types stratified by risk of PAMP contamination before and during the chronic study

Food category (by PAMP risk)	Before study	Low PAMP week	High PAMP week
<i>Food types considered to be at low risk of containing elevated levels of bacterial PAMPs</i>			
Intact meats (steaks etc.)	4.4 (1.7)	8.3 (3.4) **	0.8 (1.4) **
Fresh vegetables	12.3 (4.9)	12.2 (6.4)	2.9 (4.2) **
Fresh fruit	16.0 (8.9)	14.2 (9.0)	8.8 (7.3)
Fish	1.8 (1.9)	3.6 (3.8)	1.9 (0.5)
<i>Food types considered to be at high risk of containing elevated levels of bacterial PAMPs</i>			
Products containing minced meat	1.9 (1.5)	0.5 (1.2) *	12.3 (0.0) **
Products containing ready-chopped vegetables	1.5 (2.0)	0.4 (0.7)	5.4 (0.5) **
Ready-prepared meals stored at 4°C	0.8 (1.6)	0.0 (0.0)	7.0 (0.0) **
Chocolate	3.0 (2.1)	1.9 (2.9)	4.5 (3.9)
Cheese	2.8 (2.1)	0.8 (1.6) **	4.3 (1.2) **
Total servings of high risk foods per week	10.0 (5.3)	3.5 (3.9) **	33.4 (4.0) **

Values are expressed as servings per week, as recorded in food frequency recall questionnaires (before study) or diet diaries (low and high PAMP weeks), with SD in parentheses. The results include meals provided to the volunteers during the high PAMP arm of the study. * P<0.05; ** P<0.01 vs previous timepoint using linear mixed models with Sidak's post-test.

Figure legends

Figure 1: Effects of chronic dietary PAMP intake on inflammatory markers

Mean (\pm SE) leukocyte counts (A) and hsCRP (B) measured in fasted blood samples of healthy male volunteers (n=11), at study entry (visit 1), after a low pathogen-associated molecular pattern (PAMP) run-in diet for 7 days (visit 2), and after a further 4 days high PAMP diet (visit 3). Means were compared using linear mixed models with Sidak's post-test.

Figure 2: Effects of chronic dietary PAMP intake on regulation of body weight

Mean (\pm SE) body weight and abdominal circumference (normalised to baseline, A,B) and plasma leptin concentrations (C) measured in healthy male subjects (n=11) at study entry (visit 1), after 7 days low pathogen-associated molecular pattern (PAMP) diet (visit 2) and after 4 days high PAMP diet (visit 3). Means of untransformed (weight, waistline) or log-transformed (plasma leptin) values were compared using linear mixed models with Sidak's post-test.

Figure 3: Effects of chronic dietary PAMP intake on glucose and lipid metabolism

Mean (\pm SE) fasting serum glucose (A), triglycerides (B) and cholesterol (C) measured in healthy male subjects (n=11) at study entry (visit 1), after 7 days low pathogen-associated molecular pattern (PAMP) diet (visit 2) and after 4 days high PAMP diet (visit 3). Means were compared using linear mixed models with Sidak's post-test.

Figure 4: Effects of acute dietary PAMP intake on leukocyte inflammatory markers

Mean (\pm SE) expression of the leukocyte activation marker CD11b measured on granulocytes (A) and monocytes (B) in blood samples of healthy male volunteers (n=13) collected before ingestion of a low- or high pathogen-associated molecular pattern test meal before (0 h, fasted), or after (3 h postprandially) the test meals. CD16⁺CD14^{lo} monocytes were also quantified as a proportion of the total monocyte population (C). Means were compared by student's T-test with Bonferroni correction for multiple comparisons. * P<0.05 vs baseline. MFI, mean fluorescence intensity.