Metabolic endotoxemia: a molecular link between obesity and cardiovascular risk

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Abstract

Obesity is associated with significantly increased cardiovascular (CV) risk and mortality. Several molecular mechanisms underlying this association have been implied, among which the intestinal barrier has gained a growing interest. In experimental models of obesity, significant alterations in the intestinal barrier lead to increased intestinal permeability, favoring translocation of microbiome-derived lipopolysaccharide to the bloodstream. This has been shown to result in a two- to threefold increase in its serum concentrations, a threshold named 'metabolic endotoxemia' (ME). ME may trigger toll-like receptor 4-mediated inflammatory activation, eliciting a chronic low-grade proinflammatory and pro-oxidative stress status, which may result in high CV risk and target-organ damage. In this review, we discuss the potential molecular implications of ME on several CV risk factors, such as obesity, insulin resistance, dyslipidemia, and oxidative stress, as well as its potential impact on the development of CV target-organ disease.

Key Words
- endotoxemia
- obesity
- cardiovascular diseases

Journal of Molecular Endocrinology
(2013) **51**, R51–R64

Introduction

Cardiovascular (CV) diseases remain the leading cause of death in the western world; being estimated, they will be responsible for more than 23 million deaths in 2030 (WHO 2002). Despite the advances made in CV risk factor treatment and control, the incidence of CV disease has not significantly reduced (Pádua 2002).

Changes in nutritional status in western countries seem to contribute significantly to CV risk and mortality (Otaki 1994, Poirier & Eckel 2002). Lifestyles and eating habits promote an exponential increase in obesity, which is associated with an array of metabolic complications (dyslipidemia, insulin resistance, and type 2 diabetes mellitus (T2DM)) that foster a significant risk for CV disease (Poirier & Eckel 2002).

Obesity is associated with significantly increased CV risk and mortality (Otaki 1994, Poirier & Eckel 2002, WHO 2002). However, the molecular mechanisms underlying this association remain largely unknown. Several factors have been implied, among which the intestinal barrier has gained a growing interest (Backhed *et al.* 2004). In experimental models of obesity, significant alterations in the intestinal barrier occur (Cani *et al.* 2007*a*). In these models, structural intestinal changes lead to increased intestinal permeability, favoring translocation of microbiome-derived lipopolysaccharide (LPS) to the bloodstream (Pirlich *et al.* 2006, Cani *et al.* 2007*a*). This results in a two- to threefold increase in its serum concentrations, a threshold named 'metabolic

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DOI: 10.1530/JME-13-0079

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endotoxemia' (ME; Cani *et al*. 2007*a*). ME may trigger toll-like receptor (TLR) 4-mediated inflammatory activation, eliciting a chronic low-grade proinflammatory and pro-oxidative stress status associated with obesity, which may result in CV target-organ damage (Suganami *et al*. 2007, Puppa *et al*. 2011). ME may thus represent a molecular link between obesity and increased CV risk.

In this context and in a translational perspective, novel questions arise regarding the intricate relationship between metabolism, innate immunity, and global CV risk. A better understanding of the molecular link between the human intestinal microbiome and host's innate and inflammatory responses might thus open the way to innovative therapeutic strategies for CV risk reduction.

Intestinal changes and ME

In physiological conditions, the intestinal epithelium acts as a continuous barrier to avoid LPS translocation; however, some endogenous or exogenous events may alter this protective function (Cani *et al*. 2008).

Weight gain has been associated with a higher gut permeability and subsequent systemic exposure to mildly increased LPS circulating levels. Erridge *et al*. (2007) demonstrated that a high-fat diet promotes LPS absorption across the intestinal barrier, increasing its plasma levels by two to three times, a threshold defined as ME. These data are supported by previous studies that had also found that higher concentrations of fatty acids impair intestinal barrier integrity (Velasquez *et al*. 1993, Levels *et al*. 2001).

Two mechanisms of LPS absorption have been proposed. Ghoshal *et al*. (2009) showed in an *in vitro* model of human epithelial adenocarcinoma cells that the formation of quilomicron promotes LPS absorption. Other suggested mechanisms include LPS absorption through internalization by intestinal microfold cells (Hathaway & Kraehenbuhl 2000) and enterocytes, involving TLR4 and myeloid differentiation protein-2 (MD-2; Neal *et al*. 2006).

Moreover, some bacteria can induce and/or modulate the expression of genes involved in the barrier function in host epithelial cells (Hooper & Gordon 2001). It has been demonstrated that the introduction of a high-fat diet in mouse models resulted in a decreased expression of genes involved in the barrier function, namely zonula occludens 1 and occludin genes (Cani *et al*. 2008).

ME and innate immune response

In order to maintain the delicate relationship of mutualism with the host, intestinal bacteria need to be present above the epithelial surface or within the intestinal mucus, with those penetrating the epithelial barrier having to be immediately eliminated.

How exactly gut distinguishes between pathogens and commensal agents is a question for which the answer remains unclear. One hypothesis is that TLRs are compartmentalized in the basolateral aspects of enterocytes or inside epithelial cells (Hornef *et al*. 2003). This hypothesis suggests that a deeper bacterial–epithelial contact might be necessary in order to activate the host's innate immune response (Kelly & Conway 2005).

The starting point for innate immunity activation is the recognition of conserved structures of bacteria, viruses, and fungal components through pattern-recognition receptors (PRRs; Philpott & Girardin 2004). TLRs are PRRs that recognize microbe-associated molecular patterns (MAMPs; Turnbaugh *et al*. 2007) such as several bacterial structures of Gram-negative outer membrane (e.g., LPS) and components of Gram-positive cell wall as lipoteichoic acid or peptidoglycan (Philpott & Girardin 2004). TLRs are transmembrane proteins containing extracellular domains rich in leucine repeat sequences and a cytosolic domain homologous to the IL1 receptor intracellular domain (TIR domain) (Chow *et al*. 1999).

The LPS-sensing machinery is constituted primarily by a LPS-binding protein (LBP), a glycosylphosphatidylinositol-anchored monocyte differentiation antigen (cluster of differentiation 14 (CD14)), an accessory protein (MD-2), and TLR4 (Bosshart & Heinzelmann 2007). The primary role of LBP is the transportation of aggregates of circulating endotoxin, and the delivery of these molecules to CD14, resulting in cell activation, or to lipoproteins for hepatic clearance (Stoll *et al*. 2004). CD14 is a PRR with an important role in immunomodulation of proinflammatory signaling in response to LPS and other bacterial products (Kitchens & Thompson 2005). It is also present in a soluble form (sCD14), which derives from the secretion of CD14 or the enzymatic cleavage of the membrane form (Turnbaugh *et al*. 2007). The accessory protein MD-2, which is associated with TLR4 on the cell surface, and appears to bind to TLR4 and endotoxin, is like CD14, also known to be a critical element in this receptor complex giving it responsiveness to LPS (Nagai *et al*. 2002). Due to lack of a transmembrane domain to CD14, TLRs are required for subsequent sinalization (Chow *et al*. 1999).

The pathway is primarily activated by lipid A, a LPS MAMP from the outer membrane of Gram-negative bacteria, which binds TLR4 and its co-receptors CD14 and MD-2. The TLR4 is thus activated, causing the recruitment of adaptor molecules through interactions

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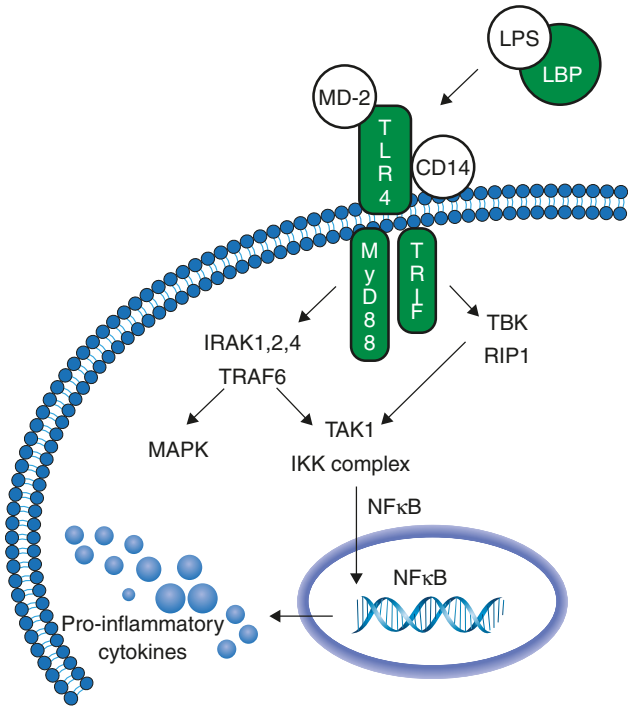

Figure 1
LPS-induced TLR4 activation induces the transcription of pro-inflammatory mediators, via the recruitment of adaptor molecules such as MyD88 and TRIF. LPS is mainly sensed through the activation of TLR4 by the LBP–LPS trigger complex. CD14 and MD-2 are critical elements in this receptor complex giving it responsiveness to LPS. TLR4 activation provokes the recruitment of four adaptor molecules, including MyD88 and TRIF. MyD88 activates the IKK complex via IRAK kinases/TRAF recruitment, lately leading to NFκB diffusion to the nucleus. TRAF6 activation also promotes MAPK activation and nuclear translocation, also inducing the transcription of pro-inflammatory cytokines. NFκB transcription also occurs in a MyD88-dependent pathway, via TRIF-mediated activation of the kinases TBK1 and RIP1. Full color version of this figure available via http://dx.doi.org/10.1530/JME-13-0079

with the TIR domain. There are four TLR4-TIR interacting adaptor molecules: MyD88; TIR domain-containing adaptor protein; TRIF-related adaptor molecule; and TRIF (TIR domain-containing adaptor inducing IFN-α) (Medzhitov 2001; Fig. 1).

MyD88 recruits IRAK4, IRAK1, and IRAK2. IRAK kinases then phosphorylate and activate the protein TRAF6, which in turn polyubiquinates the protein TAK1 as well as itself in order to facilitate binding to IKKβ. On binding, TAK1 phosphorylates IKKβ, which then phosphorylates IκB causing its degradation and allowing NFκB to diffuse into the cell nucleus and activate transcription (Lu *et al.* 2008; Fig. 1). TRAF6 activation also promotes MAPK-mediated AP-1 activation and nuclear translocation, inducing the transcription of proinflammatory cytokines. MyD88-independent intracellular pathways include TRIF-mediated activation of the kinases TBK1 and RIP1. The TRIF/TBK1 signaling complex phosphorylates IRF3 allowing its translocation into the nucleus and production of type I interferons. Moreover, RIP1 activation promotes TAK1 polyubiquination and activation and NFκB transcription in the same manner as the MyD88-dependent pathway (Werner & Haller 2007). The major proinflammatory mediators produced by the TLR4 activation in response to endotoxin (LPS) are TNFα, IL1β and IL6, which are also elevated in obese and insulin-resistant patients (Parker *et al.* 2007).

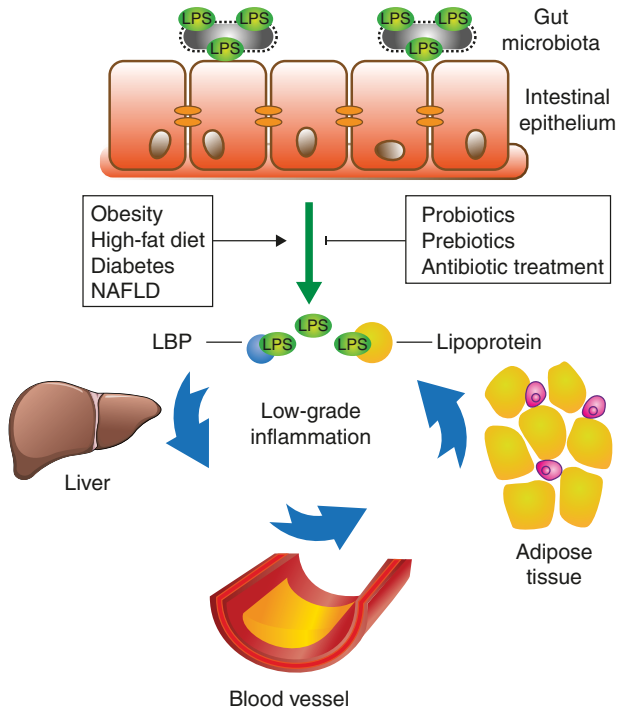

Figure 2
The gut epithelium is an efficient barrier that prevents absorption of LPS derived from Gram-negative gut microbiota. Obesity, high-fat diet, diabetes, and NAFLD are associated with higher gut permeability leading to metabolic endotoxemia. Probiotics, prebiotics, and antibiotic treatment can reduce LPS absorption and plasmatic levels. LPS in the bloodstream is transported by lipoproteins and LBP. In the liver, LPS is cleared by hepatocytes and excreted in the bile. LPS promotes hepatic insulin resistance, hypertriglyceridemia, hepatic triglyceride accumulation, and secretion of pro-inflammatory cytokines promoting the progression of fatty liver disease. In the endothelium, LPS induces the expression of pro-inflammatory, chemotactic, and adhesion molecules, which promotes atherosclerosis development and progression. In the adipose tissue, LPS induces adipogenesis, insulin resistance, macrophage infiltration, oxidative stress, and release of pro-inflammatory cytokines and chemokines.
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ME and CV risk

Given the association between ME and proinflammatory activation, several potential mechanisms have been proposed to link the gut microbiome with CV risk (Cai *et al*. 2005, Cani *et al*. 2007*a*,*b*; Fig. 2; Table 1).

ME and nutritional status

The development of obesity is the result of complex interactions between genetic and environmental factors, which are only partially understood. In this context, the gut microbiota has been recently proposed to be an environmental factor involved in the control of body weight and energy homeostasis by modulating plasma LPS levels (Backhed *et al*. 2007, Cani *et al*. 2007*a*).

Experimental evidence showed that axenic mice (raised in the absence of microorganisms) had 40% less total body fat than mice raised with normal gut microbiota (conventionalized), even if their caloric intake was higher than in conventionally raised animals (Backhed *et al*. 2004). Surprisingly, the conventionalization of axenic mice with microbiota previously harbored in nonaxenic mice was followed by a significant increase in fat mass (Backhed *et al*. 2004); moreover, mice conventionalized with microbiota from lean non-germ-free animals resulted in a fat mass gain of 40% (Backhed *et al*. 2004) and those conventionalized with the microbial community of genetically obese (*ob/ob*) mice gained up to 60%, although feed consumption was reduced in the latter (Turnbaugh *et al*. 2007). This difference in weight gain induced by conventionalization may be justified by different microbiomes and derived metabolites in lean and obese mice.

In order to understand how gut microbiota influences weight gain, germ-free and conventionalized mice were fed for 8 weeks with a high-fat, high-carbohydrate western diet. It was observed that germ-free mice gained significantly less weight and fat mass than conventionalized mice and were protected against western diet-induced insulin resistance (Backhed *et al*. 2007). This result suggests that dietary fats alone might not be sufficient to cause overweight and obesity, suggesting that a bacterially related factor might be responsible for high-fat diet-induced obesity.

Interestingly, Cani *et al*. demonstrated that after 4 weeks of high-fat feeding, mice exhibited a two- to threefold increase in circulating LPS levels, the so-called 'ME'. This was accompanied in high-fat-fed mice by a change in gut microbiota composition, with reduction in *Bifidobacterium* and *Eubacterium* spp. (Cani *et al*. 2007*a*). In line with these observations, ME was shown to be present in genetically obese leptin-deficient mice (Brun *et al*. 2007). To further understand the effects of ME on weight gain, LPS was chronically infused in wild-type mice in order to achieve 'ME'. Interestingly, these animals showed increased body weight to the same extent as a 4-week high-fat diet regimen, with visceral and subcutaneous adipose depots increasing about 40 and 30% respectively. This increase in body weight was not explained by excessive energy intake (Cani *et al*. 2007*a*).

In humans, it was also shown that meals with high-fat and high-carbohydrate content (fast-food style western diet) were able to decrease bifidobacteria levels and increase intestinal permeability and LPS concentrations (Ghanim *et al*. 2009, 2010, Fava *et al*. 2013). Moreover, it was demonstrated that, more than the fat amount, its composition was a critical modulator of ME (Laugerette *et al*. 2012). Very recently, Mani *et al*. (2013) demonstrated that LPS concentration was increased by a meal rich in saturated fatty acids (SFA), while decreased after a meal rich in n-3 polyunsaturated fatty acids (n-3 PUFA).

In fact, this effect seems to be due to the fact that some SFA (e.g., lauric and mystiric acids) are part of the lipid-A component of LPS and also to n-3 PUFA's role on reducing LPS potency when substituting SFA in lipid-A (Munford & Hall 1986, Kitchens *et al*. 1992). To clarify the mechanisms of ME-induced innate immunity activation, mice lacking TLR4 co-receptor CD14 were studied. We have shown that CD14 KO mice when exposed to a high-fat high-simple carbohydrate diet show attenuation in CV and metabolic complications of obesity compared with wild-type mice (Roncon-Albuquerque *et al*. 2008). Moreover, when chronically injected with LPS, CD14 KO mice do not show body weight gain and increased visceral and subcutaneous adipose depots, as observed in wild-type animals (Cani *et al*. 2007*a*). Taken together, these experimental results suggest a pivotal role of CD14-mediated TLR4 activation in the development of LPS-mediated nutritional changes.

Studies have also been conducted where gut microbiota was manipulated by means of antibiotic treatment. This resulted in ME reduction and attenuation of obesity, fat mass development, mRNA concentration of adipose tissue inflammatory markers, and metabolic parameters of obesity in both high-fat-fed and *ob/ob* mice (Cani *et al*. 2008, Membrez *et al*. 2008). Similar results were observed when an endotoxin inhibitor was administered for 4 weeks to *ob/ob* mice (Cani *et al*. 2008).

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Table 1 Most relevant studies on the effects of metabolic endotoxemia on CV risk

References	Title	Main results and conclusions	Limitations
Animal model studies			
Feingold *et al.* (1992)	Endotoxin rapidly induces changes in lipid metabolism that produce hypertriglyceridemia: low doses stimulate hepatic triglyceride production while high doses inhibit clearance	Low doses of endotoxin induce hypertriglyceridemia in rodents by increasing the hepatic secretion of triglyceride, hepatic *de novo* fatty acid synthesis, and lipolysis. High doses of LPS produce hypertriglyceridemia by decreasing lipoprotein catabolism. Administration of TNF antibodies or IL1 receptor antagonist did not prevent the increase in serum triglyceride levels induced by LPS	Unclear causal relationship: the increase in serum triglycerides could be a protective response by the host against the toxic effects of LPS Lipoprotein metabolism differs markedly in humans when compared with rodents
Lehr *et al.* (2001)	Immunopathogenesis of atherosclerosis: endotoxin accelerates atherosclerosis in rabbits on a hypercholesterolemic diet	Rabbits on a hypercholesterolemic diet that receive repeated i.v. injections of endotoxin exhibited significantly accelerated atherosclerosis compared with animals of the hypercholesterolemic control group after 8 weeks. Triglycerides and LDL and HDL levels were similar in the two groups of animals	Endotoxin significantly accelerated atherosclerosis by increasing aortic lesion area and lesion volume but not lesion thickness
Cani *et al.* (2007*a*)	Metabolic endotoxemia initiates obesity and insulin resistance	Four-week high-fat diet chronically increased plasma LPS concentration two to three times Metabolic endotoxemia induced through s.c. infusion of LPS provokes insulin resistance, liver, body, and adipose tissue weight gain to a similar extent as in high-fat-fed mice Liver insulin resistance, markers of inflammation, and liver triglyceride content were increased in LPS-infused mice *Cd14* mutant mice resisted most of the LPS and high-fat diet-induced features of metabolic diseases	Insulin resistance in LPS-infused mice was only detected in liver, whereas high-fat diet mice developed whole-body, but not liver, insulin resistance
Brun *et al.* (2007)	Increased intestinal permeability in obese mice: new evidence in the pathogenesis of nonalcoholic steatohepatitis	Genetically obese mice display enhanced intestinal permeability leading to higher circulating levels of inflammatory cytokines and portal endotoxemia compared with lean control mice Hepatic stellate cells of obese mice showed higher membrane *Cd14* mRNA levels and more endotoxin-induced pro-inflammatory and fibrogenic responses than lean animals	
Cani *et al.* (2007*b*)	Selective increases in bifidobacteria in gut microflora improve high-fat diet-induced diabetes in mice through a mechanism associated with endotoxemia	High-fat feeding significantly increased endotoxemia, which was normalized to control levels in mice treated with prebiotic High-fat feeding reduced intestinal bacteria and *Bifidobacterium* spp. The levels of bifidobacteria were restored by the introduction of a prebiotic. Endotoxemia was negatively correlated with *Bifidobacterium* spp. In high-fat mice treated with prebiotics, *Bifidobacterium* spp. is positively correlated with an improved glucose tolerance, glucose-induced insulin secretion, and normalized inflammatory tone, decreasing endotoxemia, plasma, and adipose tissue proinflammatory cytokines Prebiotic supplementation also improves body weight gain and energy intake, reduces fat mass development, and increases colonic glucagon-like peptide-1 precursor	The prebiotic doses used in animal studies are not directly transposable to human nutrition No relationship was found between endotoxemia and any other bacterial group besides *Bifidobacterium* spp.

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DOI: 10.1530/JME-13-0079

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Table 1 *Continued*

References	Title	Main results and conclusions	Limitations
Cani *et al*. (2008)	Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice	Changes in gut microbiota by antibiotic treatment reduce metabolic endotoxemia and the cecal content of LPS in high-fat-fed and *ob/ob* mice. This effect was correlated with reduced glucose intolerance, body weight gain, fat mass development, lower inflammation, oxidative stress, and macrophage infiltration in visceral adipose tissue. High-fat feeding also increased intestinal permeability and reduced the expression of genes coding for proteins of the tight junctions. The absence of *Cd14* in *ob/ob* mutant mice provokes the same metabolic and inflammatory effects of antibiotics	
Human studies			
Wiedermann *et al*. (1999)	Association of endotoxemia with carotid atherosclerosis and cardiovascular disease: prospective results from the Bruneck study	Subjects with higher levels of endotoxin concentrations face a threefold risk of incident atherosclerosis. This risk was most pronounced in subjects with chronic infections and in current and ex-smokers. Smokers with low endotoxin levels and nonsmokers did not differ in their atherosclerosis risk, whereas smokers with high endotoxin levels almost invariably developed new lesions	Strict inclusion criteria: only included people in the sixth and eighth decade of life
Niebauer *et al*. (1999)	Endotoxin and immune activation in chronic heart failure; a prospective cohort study	Raised concentrations of endotoxin and cytokines are found in patients with chronic heart failure during acute edematous exacerbation. Intensified diuretic treatment normalizes endotoxin concentrations, suggesting that endotoxin may trigger immune activation in patients with chronic heart failure during edematous episodes	Unclear causal relationship: after short-term diuretic treatment, although endotoxin concentrations have decreased, cytokines remained raised
Agwunobi *et al*. (2000)	Insulin resistance and substrate utilization in human endotoxemia	LPS administration induces fever, tachycardia, and hypotension in healthy human volunteers. Glucose utilization increased abruptly 120 min after LPS administration but declined progressively leading to insulin resistance after 420 min of LPS administration. LPS also induced significant increases in plasma cortisol, glucagon, GH, IL6, and TNF concentrations	Small sample. The changes in glucose utilization could be attributed to the hemodynamic effect of LPS; it might not represent a chronic effect of endotoxemia, but just an acute response to LPS infusion
Anderson *et al*. (2007)	Innate immunity modulates adipokines in humans	LPS induced fever, blood and adipose TNF, and IL6 release and insulin resistance. Also doubled the leptin: soluble leptin receptor ratio, and increased the plasma resistin in healthy humans Total adiponectin levels and low- and high-molecular weight adiponectin complexes were unaltered by LPS treatment, and whole blood mRNA for adiponectin receptors 1 and 2 was suppressed	Small sample
Creely *et al*. (2007)	Lipopolysaccharide activates an innate immune system response in human adipose tissue in obesity and type 2 diabetes	Subjects with type 2 diabetes had 76% higher circulating LPS, and LPS is correlated with insulin in controls	

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Table 1 *Continued*

References	Title	Main results and conclusions	Limitations
		Treatment of human abdominal subcutaneous adipocytes with LPS caused a significant increase in secretion of TNFα and IL6. The protein expression of TLR2, TRAF6, and NFκB was also increased in LPS-treated adipocytes	
Erridge *et al.* (2007)	A high-fat meal induces low-grade endotoxemia: evidence of a novel mechanism of postprandial inflammation	In humans after a high-fat meal with or without cigarettes, an increase in endotoxin concentrations and a reduction in endotoxin neutralization capacity are observed. Human monocytes were responsive to transient or low-dose exposure to endotoxin	Small sample. CRP did not increase over the duration of the study
		Low-grade endotoxemia may contribute to the postprandial inflammatory state and could represent a novel potential contributor to endothelial activation and the development of atherosclerosis	The 4-h duration of the study may have been insufficient to witness increases in inflammatory markers Aortic endothelial cells were unresponsive to transient or low-dose exposure of endotoxin
Amar *et al.* (2008)	Energy intake is associated with endotoxemia in apparently healthy men	Endotoxemia was independently associated with energy intake but not fat intake	The analyses of LPS were performed in a semiquantitative way, with the lower limit of LPS plasma detection of 9 U/ml
		No significant relation was observed between weight, BMI, insulin, glycemia, CV disease risk factors, carbohydrate or protein intakes, and plasma LPS concentration in humans	
Miller *et al.* (2009*a*)	Ethnic and sex differences in circulating endotoxin levels: a novel marker of atherosclerotic and cardiovascular risk in a British multi-ethnic population	Age-adjusted endotoxin levels were lower in women than in men, and were highest in south Asians and lowest in individuals of African origin than in whites	
		Endotoxin levels were positively associated with waist, waist:hip ratio, total cholesterol, serum triglycerides, and serum insulin levels and negatively associated with serum HDL-cholesterol. Serum hs-CRP and plasma sCD14 varied by ethnic group but were not associated with endotoxin	

Other manipulations of gut microbiota include the use of pre- and probiotics. Prebiotics show efficacy in protecting against high-fat diet-induced ME, also reducing body weight gain and fat mass (Cani *et al*. 2007*b*). This suggests a link between gut microbiota, western diet, and obesity and indicates that gut microbiota manipulation can beneficially affect the host's weight and adiposity. Although the data derived from animal models seem to support this link, the cause–effect relationships remain unclear and a limited number of *in vivo* trials have been performed so far. In one of the few cross-sectional studies performed in humans, endotoxemia was independently associated with energy intake but not fat intake in a multivariate analysis (Amar *et al*. 2008).

On the other hand, epidemiological studies strongly support that obesity and hypercholesterolemia paradoxically improve survival in cardiac cachexia, and thus, it would not be surprising that a hypercaloric and hyperproteic western diet could have some benefits in these cachectic patients (Song *et al*. 2006). This hypothesis has been tested in an animal model of monocrotaline-induced cardiac cachexia, being shown that in the group that consumed a western-type diet, the extent of myocardial remodeling and apoptosis were lower when compared with the group consuming a normal diet (Lourenco *et al*. 2011). The western-type diet group also had a more favorable inflammatory profile (lower myocardial NFκB transcription factor activity, endothelin-1 and cytokine overexpression and concentrations). Surprisingly, western-type diet attenuated cardiac cachexia and inflammation and improved survival, suggesting a relationship between the diet, inflammation, and CV risk in cachexia (Lourenco *et al*. 2011).

ME and insulin resistance

It has been proposed that ME and dietary fats might also impair carbohydrate metabolism up to insulin resistance and, lately, T2DM.

In vitro studies showed that preadipocytes mediate LPS-induced insulin resistance in primary cultures of newly differentiated human adipocytes. Chung *et al*. (2006) demonstrated *in vitro* that endotoxemia activates pro-inflammatory cytokine/chemokine production via NFκB and MAPK signaling in preadipocytes and decreased peroxisome proliferator-activated receptor γ activity and insulin responsiveness in adipocytes.

In order to study the relationship between ME and insulin resistance, LPS was continuously infused for 1 month in wild-type mice with a s.c. minipump to achieve 'ME'. These animals developed the same metabolic abnormalities as those usually induced by a high-fat diet, including hyperglycemia, hyperinsulinemia, and hepatic insulin resistance (Cani *et al*. 2007*a*). Moreover, CD14 KO mice were resistant to high-fat diet and chronic LPS infusion, showing hyperinsulinemia and insulin resistance significantly later when compared with wild-type animals. Of note, intrahepatic accumulation of triglycerides was totally blunted in CD14 KO mice. CD14 KO mice also showed hypersensitivity to insulin when fed a normal diet, suggesting a role for CD14 in the modulation of insulin sensitivity even in physiological conditions (Cani *et al*. 2007*a*).

Interventions manipulating the gut microbiome might also affect glycemic metabolism. Backhed *et al*. (2004) noticed that gut colonization of germ-free mice with cecum-derived microbes resulted in insulin resistance with no impact in chow consumption or energy expenditure.

A few studies in humans have also related ME to impaired glucidic metabolism. Creely *et al*. (2007) showed that T2DM patients have mean values of LPS that are 76% higher than healthy controls. Plus, van der Crabben *et al*. showed that even low doses of LPS are able to induce changes in glucose uptake in lean humans, which presented enhanced insulin sensitivity in the first few hours after the injection, followed later by its significant reduction. Furthermore, circulating insulin and glucose levels were increased (Anderson *et al*. 2007, van der Crabben *et al*. 2009).

LPS exposure resulted in reduced hepatic glucose production and improved glucose clearance in healthy volunteers (van der Crabben *et al*. 2009, Raetzsch *et al*. 2009). This effect might be dependent on the LPS-induced release of glucagon, GH and cortisol, which inhibit glucose uptake, both peripheral and hepatic (Agwunobi *et al*. 2000).

Finally, LPSs also seem to induce ROS-mediated apoptosis in pancreatic cells. Du *et al*. showed that ROS-mediated LPS-induced apoptosis in insulin-secreting cells from a rat pancreatic cell line (ins-1) occurs in both dose- and time-dependent manners. This effect may lead to subsequent defective pancreatic cell function and decreased insulin secretion (Du *et al*. 2012).

ME and dyslipidemia

Recent evidence has been linking ME with dyslipidemia, increased intrahepatic triglycerides, development, and progression of alcoholic and nonalcoholic fatty liver disease (NAFLD; Manco *et al*. 2010).

http://jme.endocrinology-journals.org
DOI: 10.1530/JME-13-0079

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LPS is transported in the bloodstream by its specific transport protein (LBP) and by lipoproteins to hepatocytes (Netea *et al*. 2004). The hepatocytes, rather than hepatic macrophages, are the cells responsible for its clearance, being ultimately excreted in bile (Read *et al*. 1993). All the subclasses of plasma lipoproteins can bind and neutralize the toxic effects of LPS, both *in vitro* (Eichbaum *et al*. 1991) and *in vivo* (Harris *et al*. 1990), and this phenomenon seems to be dependent on the number of phospholipids in the lipoprotein surface (Levels *et al*. 2001). LDL seems to be involved in LPS clearance, but this antiatherogenic effect is outweighed by its proatherogenic features (Stoll *et al*. 2004).

LPS produces hypertriglyceridemia by several mechanisms, depending on LPS concentration. In animal models, low-dose LPS increases hepatic lipoprotein (such as VLDL) synthesis, whereas high-dose LPS decreases lipoprotein catabolism (Feingold *et al*. 1992, Sanz *et al*. 2008).

Some authors have pointed out that the high capacity of LPS binding to HDL suggests that HDL might provide additional protection against LPS-induced inflammation, like in sepsis or in the proatherogenic and diabetogenic effect observed in 'ME' (Barcia & Harris 2005). Reinforcing this hypothesis, it was observed that an infusion of reconstituted HDL 3.5 h before a LPS challenge (4 ng/kg) markedly reduced LPS-induced release of TNFα, IL6, and IL8 in humans (Pajkrt *et al*. 1996). Inversely, in a hypolipidemic rat model, LPS produced a three- to fivefold greater increase in TNFα levels when compared with controls (Feingold *et al*. 1995).

When a dose of LPS similar to that observed in ME was infused in humans, a 2.5-fold increase in endothelial lipase was observed, with consequent reduction in total and HDL. This mechanism may explain low HDL levels in 'ME' and other inflammatory conditions such as obesity and metabolic syndrome (Stoll *et al*. 2004).

It is known that the high-fat diet and the 'ME' increase intrahepatic triglyceride accumulation, thus synergistically contributing to the development and progression of alcoholic and NAFLD, from the initial stages characterized by intrahepatic triglyceride accumulation up to chronic inflammation (nonalcoholic steatohepatitis), fibrosis, and cirrhosis (Manco *et al*. 2010). The increase in fatty acids in hepatocytes enhances the hepatic expression of TLR4 and TLR2, as well as its co-receptors CD14 and MD-2 (Maher *et al*. 2008). This favors activation by SFAs, LPSs, or both, enhancing the progression from fatty liver to steatohepatitis (Mencin *et al*. 2009). On the other hand, LPS activates Kupffer cells leading to an increased production of ROS and pro-inflammatory cytokines like TNFα. This mechanism has been shown to promote the progression of fatty liver disease to steatohepatitis (Hritz *et al*. 2008).

Recently, it has been demonstrated that patients with NAFLD have a reduced expression of the tight junction protein zonula occludens 1, thus presenting increased intestinal permeability (Miele *et al*. 2009). It was also found that these patients' degree of intestinal permeability was proportional to their degree of steatosis. These changes in intestinal permeability have also been shown to promote ME (Miele *et al*. 2009).

The administration of prebiotics and probiotics in various models of liver disease, including NASH and LPS-induced liver failure resulted respectively in the inhibition of the inflammatory activity and improvement of NAFLD (Li *et al*. 2003) and in the prevention of hepatic damage (Ewaschuk *et al*. 2007).

ME, low-grade inflammation, and oxidative stress

Low-grade inflammation has been linked to CV risk, with several studies pointing out that increased levels of pro-inflammatory cytokines (C-reactive protein, soluble vascular cell adhesion molecule 1, and intercellular adhesion molecule-1) are associated with higher CV mortality (Jager *et al*. 1999, Becker *et al*. 2002, Danesh *et al*. 2004).

ME seems to participate in this molecular pathway, acting as a trigger to the low-grade inflammatory response. In a previously described animal model, Cani *et al*. changed gut microbiota by means of antibiotic treatment to demonstrate that changes in gut microbiota could be responsible for the control of ME and low-grade inflammation. The authors first showed that high-fat diet mice presented with ME, which positively and significantly correlated with plasminogen activator inhibitor (PAI-1), IL1, TNFα, STAMP2, NADPHox, MCP-1, and F4/80 (a specific marker of mature macrophages) mRNAs (Cani *et al*. 2008). Subsequently, in a different interventional study, it was also shown that prebiotic administration reduces intestinal permeability to LPS in obese mice and is associated with decreased systemic inflammation when compared with controls. Changing the gut microbiota through an intervention was associated with significantly reduced *Pai-1*, *Cd68*, *Nadph* oxidase (*NadpHox*), and inducible nitric oxide synthase mRNA concentrations and tended to decrease *Tlr4* and *Tnfα* mRNA concentrations (Cani *et al*. 2008).

LPS also seems to affect oxidative stress, which has also been implied in CV morbidity and mortality. We have shown that allelic variants of (Cu–Zn)SOD, an enzyme

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belonging to the superoxide dismutase family and that play a major role in detoxification of ROS and protection against oxidative stress, are associated with increased risk of death from CV causes (sudden death, fatal myocardial infarction, or stroke) (Neves *et al*. 2012). Gibbs *et al*. (1992) demonstrated that lung endothelial MnSOD (both mRNA and protein) was increased by LPS treatment in LPS-sensitive mice, but not in LPS-resistant mice. Conversely, TNFα increased MnSOD mRNA levels in both models, LPS-sensitive and resistant. On the other hand, LPS exposure did not affect either macrophage or endothelial cell Cu/ZnSOD mRNA/protein levels (Gibbs *et al*. 1992). These findings suggest that the mutation that shapes LPS susceptibility probably exerts its effect in a cell-specific way (Gibbs *et al*. 1992). Tsan *et al*. (2001) additionally demonstrated that induction of MnSOD by LPS is mediated by mCD14 and TLR4 in murine macrophages.

Cani *et al*. also found that high-fat diet mice presented with not only ME but also higher levels of inflammatory markers, oxidative stress, and macrophage infiltration markers. Plus, positive and significant correlations were found among these variables. This suggests that important links between gut microbiota, ME, inflammation, and oxidative stress are implicated in a high-fat diet situation (Cani *et al*. 2008). Plus, the authors showed that the antibiotic treatment completely abolished these effects, normalizing not only the increase in inflammatory markers but also normalizing lipid peroxidation in the visceral depots and the oxidative stress markers STAMP2 and NADPHox on visceral and subcutaneous adipose depots. The mRNA concentrations of chemokines MCP-1 and F4/80 were increased in high-fat mice and totally normalized by the antibiotic treatment.

These results are also supported by previous studies that have described that high-fat feeding is associated with adipose tissue macrophage infiltration (F4/80-positive cells) and increased levels of chemokine MCP-1, suggesting a strong link between ME, proinflammatory status, oxidative stress, and, lately, increased CV risk (Weisberg *et al*. 2003, Kanda *et al*. 2006).

ME and CV disease

As described above, ME relates to several known CV risk factors and lately promotes low-grade chronic inflammation and oxidative stress, two recognized factors of CV disease. Thus, it is not surprising that ME is also associated with real target-organ CV disease. Discussed as follows, LPS has been shown to promote atherosclerosis, a hallmark of CV disease.

The effect of LPS on the CV system was demonstrated in patients with chronic kidney disease (CKD), in which ME correlated with the CV disease burden (systemic inflammation and cardiac injury) and with a higher risk of mortality. The authors suggest that CKD patients, namely those undergoing hemodialysis, experience systemic circulatory stress and recurrent regional ischemia that contributes to increased LPS translocation through the intestinal barrier (McIntyre *et al*. 2011).

In order to specifically demonstrate the effect of LPS on the development of atherosclerosis, Lehr *et al*. showed that LPS-treated animals exhibited significantly accelerated atherosclerosis compared with control animals, using an animal model of hypercholesterolemic rabbits, which received either repeated i.v. injections of endotoxin or a self-limiting cutaneous *Staphylococcus aureus* infection. Endotoxin-treated animals exhibited significantly accelerated atherosclerosis compared with control animals (Lehr *et al*. 2001).

In humans, the Bruneck study was the first specifically assessing the impact of subclinical endotoxemia on the development of carotid atherosclerosis. The authors showed that markers of systemic inflammation such as circulating bacterial endotoxin were elevated in patients with chronic infections and were strong predictors of increased atherosclerotic risk (Kiechl *et al*. 2001).

Several molecular mechanisms explain the role of LPS in atherosclerotic plaque formation and progression. As previously described, under endotoxemic conditions, endothelial cells release proinflammatory, chemotactic, and adhesion molecules, drawing T lymphocytes to form the fibrous cap of atherosclerotic lesions (Larsen *et al*. 1989) and inducing monocyte transmigration, adhesion on the endothelial monolayer, differentiation into macrophages, and plaque formation (Gerszten *et al*. 1999). Endotoxin can also induce activation and up-regulation of other molecules involved in cell–cell and cell–matrix interaction and communication, such as β2-integrins, selectins, platelet/endothelial cell adhesion molecule-1, and platelet-activating factor (Shen *et al*. 1998).

Plus, Wiesner *et al*. (2010) suggested that cooperative engagement of NFκB transcription factors by mmLDL and LPS results in additive/synergistic upregulation of proinflammatory genes in macrophages, thus constituting a mechanism of increased transcription of inflammatory cytokines within atherosclerotic lesions.

As a TLR4 ligand, LPS has been suggested to induce atherosclerosis development and progression, via a TLR4-mediated inflammatory state. Michelsen *et al*. (2004) showed that mice lacking TLR4 presented with

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reduced aortic atherosclerosis, lower levels of circulatory proinflammatory cytokines, and decreased lipid content in the plaques. On the other hand, Miller *et al.* (2009*b*) used a zebrafish model of early stages of atherosclerosis, which demonstrated that lack of TLR resulted in a significant decrease in the *in vivo* rate of lipidic accumulation on macrophages present on vascular lesions.

In humans, *TLR4* mutations have been shown to be associated with a decreased response to inhaled LPS (Arbour *et al.* 2000). Plus, a reduced risk of carotid artery atherosclerosis development (Kiechl *et al.* 2002) and the appearance of acute coronary events have been observed in association with its Asp299Gly polymorphism (Ameziane *et al.* 2003, Boekholdt *et al.* 2003).

Conclusion

The intestinal microbiome gained growing interest as a modulator of inflammation and oxidative stress, factors increasingly implicated in the pathophysiology of CV disease. According to the actual evidence, some authors have suggested that it might have itself a role as a CV risk marker. Nevertheless, several questions remain to be answered (Box 1).

First, the factors influencing LPS translocation are not completely understood and might be addressed in future studies. As discussed before, high-fat and high-carbohydrate content (fast-food style western diet) increase intestinal permeability and LPS concentrations. Thus, it would not be surprising that other characteristics of dietary components might also play a role in LPS translocation (pH, salt or sucrose content, other dietary nutrients). Very recently, it has been found that high levels of trimethylamine N-oxide, a product of phosphatidylcholine digestion by intestinal bacteria, are associated with increased risk of incident major CV events (Tang *et al.* 2013). The study and modulation of other dietary component effects might lead to novel additional research lines on this field.

Box 1 Future research lines

1) Other factors influencing LPS absorption/translocation
   a. Environmental factors
      i. Other dietary characteristics and components (e.g., pH, salt content, sucrose content, other dietary nutrients); therapeutic options modulating these components
   b. Genetic factors
2) Identification of other LPS-mediated pathways leading to CV disease
3) Epidemiological studies

Plus, genetic determinants may also play a role in LPS translocation; in intestinal bowel disease, some genetic factors (such as mutation of *CARD15*) are involved in the impairment of intestinal barrier function and high mucosal permeability (Schreiber 2006). In CV disease, mutations leading to increased gut permeability can also lead to a higher probability of developing ME in response to the environmental factors such as nutrition. This could explain why CV disease develops differently in patients exposed to the same environmental conditions, thus integrating the genetic environmental concepts.

Finally, the relationship between ME and CV disease shall be further clarified by epidemiologically robust evidence. The Bruneck study was the first to evoke a clinical association between LPS levels and CV risk (Wiedermann *et al.* 1999). More recently, the Wandsworth Heart and Stroke Study showed an ethnic influence on LPS levels, which increased from black Africans to caucasians and to south Asians (Miller *et al.* 2009*a*). The authors pointed out that this increase was compatible with ethnic differences in CV risk, as an increase in the number of components of the metabolic syndrome and in 10-year CV risk (Framingham score) was also observed. Although compelling evidence suggests a molecular link between ME and CV risk, more powerful epidemiological studies are needed to clarify the strength of this association.

Finally, research lines addressing the understanding of LPS-mediated pathways leading to CV disease may also lead to the identification of other molecules that also contribute to this disease. A better understanding of these molecular mechanisms may unravel novel and innovative therapeutic approaches to reduce CV risk.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

Funding
This work was supported by Portuguese Foundation for Science and Technology Grants PEst-C/SAU/UI0051/2011 and PTDC/SAU-MET/116119/09 through the Cardiovascular R&D Unit and SFRH/BD/52036/2012.

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Received in final form 23 July 2013
Accepted 12 August 2013
Accepted Preprint published online 12 August 2013

http://jme.endocrinology-journals.org
DOI: 10.1530/JME-13-0079

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