

## Irish Section Postgraduate Symposium

# Pathogenic obesity and nutraceuticals

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Over a decade of intense research in the field of obesity has led to the knowledge that chronic, excessive adipose tissue expansion leads to an increase in the risk for CVD, type 2 diabetes mellitus and cancer. This is primarily thought to stem from the low-grade, systemic inflammatory response syndrome that characterises adipose tissue in obesity, and this itself is thought to arise from the complex interplay of factors including metabolic endotoxaemia, increased plasma NEFA, hypertrophic adipocytes and localised hypoxia. Plasma concentrations of vitamins and antioxidants are lower in obese individuals than in the non-obese, which is hypothesised to negatively affect the development of inflammation and disease in obesity. This paper provides a review of the current literature investigating the potential of nutraceuticals to ameliorate the development of oxidative stress and inflammation in obesity, thereby limiting the onset of obesity complications. Research has found nutraceuticals able to positively modulate the activity of adipocyte cell lines and further positive effects have been found in other aspects of pathogenic obesity. While their ability to affect weight loss is still controversial, it is clear that they have a great potential to reverse the development of overweight and obesity-related comorbidities; this, however, still requires much research especially that utilising well-structured randomised controlled trials.

### Nutraceuticals: Polyphenols: Obesity: Inflammation: Adipose tissue

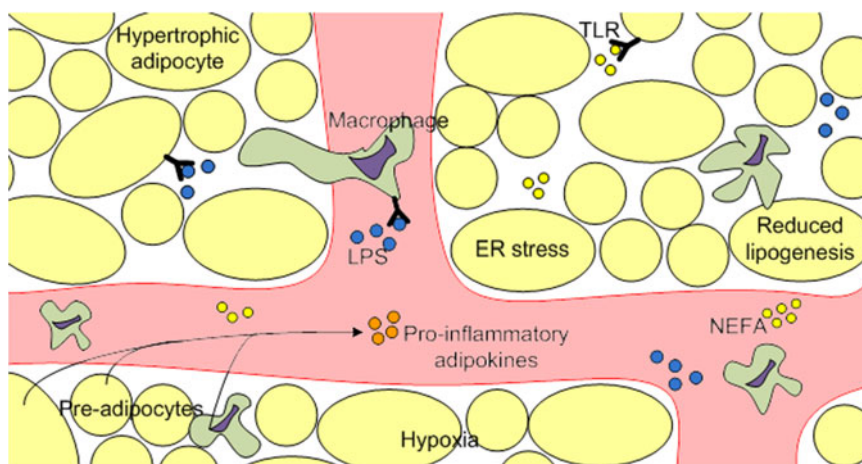
Obesity is considered the epidemic of the 21st century and worldwide in 2008 approximately 1.5 billion adults were classed as overweight, with a third of these classed as obese, numbers which are expected to increase over the next 5–10 years<sup>(1)</sup>. Having significant associations with CVD, type 2 diabetes mellitus (T2DM) and cancer<sup>(2)</sup>, obese individuals have been found to have a substantially higher use of healthcare services than the non-obese<sup>(3)</sup>; therefore, as prevalence increases, so will the burden on healthcare systems. It is now widely recognised that obesity is characterised by a chronic, low-grade, systemic inflammatory response stemming from enlarged adipose tissue (AT) depots, and this is thought to be involved in the development of obesity-related pathologies<sup>(4)</sup>. Furthermore, plasma levels of vitamins and antioxidants are lower in the obese<sup>(5,6)</sup> and an inverse relationship has been shown

between serum total antioxidant capacity and waist circumference<sup>(7)</sup>, research also indicates the modulatory effects of vitamins and antioxidants on the immune system<sup>(8)</sup> and it may be that reduced levels have a role in the development of inflammation and ultimately disease, in obesity.

Current pharmacotherapy for obesity primarily involves Orlistat and Sibutramine; however, their side effects, combined with the uncertain long-term effects associated with their use, have prompted research into alternatives. The term ‘nutraceuticals’ was originally defined as ‘a food (or part of a food) that provides medical or health benefits, including the prevention and/or treatment of disease’<sup>(9)</sup>. This term encompasses substances that are not traditionally recognised nutrients (e.g. vitamins and minerals), but that have been found to have positive physiological effects on

**Abbreviations:** AT, adipose tissue; C/EBP, cytidine-cytidine-adenosine-adenosine-thymidine-enhancer-binding protein; EGCG, epigallocatechin-3-O-gallate; ER, endoplasmic reticulum; HIF, hypoxia-inducible factor; LPS, lipopolysaccharide; sNEFA, saturated NEFA; T2DM, type 2 diabetes mellitus; TLR, Toll-like receptor; UPR, unfolded protein response.

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**Fig. 1.** (Colour online) The development of pathogenic adipose tissue in obesity is a complex interplay of many factors. LPS, lipopolysaccharide; ER, endoplasmic reticulum; TLR, Toll-like receptors.

the body<sup>(10)</sup> and includes plant phytochemicals and phenolics. There is currently no coherent, international definition of a nutraceutical; however, over the last decade, there has been a surge in their marketing, sales and research and as a result there is a requirement for governing bodies to define this area in order to uphold regulatory laws. It is generally regarded that a nutraceutical is a food component consumed in a unit dose form such as tablets, capsules or liquids and includes those from botanical sources<sup>(10)</sup>. Polyphenols are now known to act as signalling molecules and have been found to affect cellular function and modulate gene expression in relation to cancer<sup>(11)</sup> and neurodegenerative disease<sup>(12)</sup>. However, research into the potential use of nutraceuticals in the treatment of chronic inflammatory diseases such as obesity has only recently been undertaken and as such there is still much to be investigated.

### Adipose tissue in obesity

AT comprises mature adipocytes and non-fat stromovascular cells including fibroblasts, endothelial cells, pre-adipocytes and tissue resident macrophages<sup>(13)</sup>. Originally thought of as connective tissue where excess energies were stored as TAG, in the last decade, the endocrine function of AT has been demonstrated and it is now known to produce numerous products collectively called 'adipokines' involved in energy metabolism, inflammatory response and cardiovascular activity<sup>(14,15)</sup>. The two major adipokines secreted are leptin and adiponectin.

Leptin is a protein product of the *ob* gene initially known for its role in regulating appetite<sup>(16)</sup>. In obesity, circulating levels are increased in proportion to fat mass<sup>(17,18)</sup>, possibly due to increased expression of the *ob* gene and elevated leptin secretion<sup>(19,20)</sup>. Women have higher-serum leptin concentrations than men regardless of percent body fat or fat mass<sup>(21,22)</sup>, gender differences also seen in obese children<sup>(23)</sup>. Leptin shares homology with some pro-inflammatory cytokines and is indeed regarded as such; the leptin receptor is expressed by immune cells<sup>(24,25)</sup> and in human subjects circulating levels

of leptin are positively associated with those of C-reactive protein, a marker of inflammation<sup>(26)</sup>. Adiponectin is regarded as anti-inflammatory and also regulates insulin sensitivity<sup>(14)</sup>. Normal-weight individuals have high circulating levels of adiponectin; however, this decreases as adiposity increases<sup>(27)</sup> and levels also negatively correlate with insulin resistance, T2DM and CVD<sup>(28)</sup>. Adiponectin synthesis is increased by weight loss and thiazolidinediones, used in the treatment of T2DM, conversely TNF $\alpha$  and IL-6 reduce adiponectin synthesis<sup>(29,30)</sup>. In human subjects, there is an inverse correlation between plasma C-reactive protein levels and adiponectin<sup>(26,31,32)</sup>. Furthermore, in mice, adiponectin gene knockdown results in diet-induced insulin resistance<sup>(33)</sup> and an increased inflammatory response to dextran sulfate sodium-induced colitis<sup>(34)</sup> and tissue ischaemia<sup>(35)</sup>.

Chronic excessive energy intake leads to AT expansion through both hypertrophy and hyperplasia, and in adults hypertrophy seems to dominate<sup>(36,37)</sup>. In some adults, however, hyperplasia can be predominate, which is thought to result in the development of a metabolically benign form of obesity<sup>(38)</sup>. Dysregulated secretion of adipokines occurs as obesity develops and there is a general shift in adipokine and cytokine production towards a pro-inflammatory composition. This is thought to be mediated by the activation of tissue-resident macrophages in addition to a significant infiltration of other immunocytes, predominantly macrophages<sup>(39)</sup> although the triggering factors for this are, as yet, unclear. However, the main factors implicated in the initiation of inflammation and oxidative stress in obesity are thought to be metabolic endotoxaemia, increased plasma NEFA, hypertrophic adipocytes and increased AT hypoxia<sup>(13)</sup> (Fig. 1). These factors do not develop in isolation and there are most likely mechanisms involved which act to feed-forward these processes.

### Metabolic endotoxaemia

It is now well known that there is a post-prandial inflammatory response, especially following meals high in fat

and carbohydrate<sup>(40)</sup>. Macronutrient intake has also been shown to induce pro-inflammatory changes in the immunocytes of normal-weight subjects, a response that is greater and more prolonged in the obese<sup>(41)</sup>, and which is not seen following consumption of a meal rich in fruit and fibre<sup>(42)</sup>. Recent research suggests that this may be due to the presence of Toll-like receptor (TLR) stimulants, such as bacterial lipopeptides and lipopolysaccharides (LPS), which have been found primarily in meat and processed food products even though they are fit for consumption<sup>(43)</sup>. Microbiota of the gut has recently been identified as a triggering factor of inflammation in obesity, and indeed for the development of obesity itself. Germ-free mice, which contain no gut microbiota, have 42% less total body fat than conventionally raised mice; however, re-colonisation with microbiota from normal mice, increased their total body fat content by 57%<sup>(44)</sup>. It is thought that microbiota aid the absorption of monosaccharides and induce hepatic lipogenesis, promoting energy storage with a subsequent increase in adiposity<sup>(44)</sup>.

In trying to further identify the causative factors for post-prandial inflammation, research indicates that it may be absorption of the endotoxin LPS, released during the death of Gram-negative bacteria within the gut; this is also proposed to have a role in the development of obesity and inflammation<sup>(45)</sup>. In mice, a high-fat or high-carbohydrate meal increases plasma endotoxin levels<sup>(45)</sup>. Similarly, in human subjects, consumption of a high-fat meal has been found to increase plasma levels of endotoxin<sup>(42,46)</sup> and positive correlations have been found between energy or fat intake and plasma endotoxin levels in healthy men<sup>(47)</sup>. One study has further shown there to be a difference between fatty acids in increasing plasma LPS concentrations. Genetically obese JCR:LA-cp (James C Russell corpulent) rat fed a diet containing either 5% or 10% PUFA had significantly lower levels of LPS-binding protein than those on the isoenergetic, lipid-balanced control diets<sup>(48)</sup>. It would be interesting to elucidate if PUFA have similar properties in human subjects.

This significant increase in plasma LPS in response to diet composition has been termed as 'metabolic endotoxaemia'<sup>(45)</sup>. Plasma levels of LPS-binding protein, a marker of endotoxaemia, are significantly higher in obese human subjects and are associated with metabolic syndrome and T2DM<sup>(49)</sup>. Research in mice has found that a high-fat or high-carbohydrate diet increases the Gram-negative bacteria population of the gut along with increasing plasma levels of LPS<sup>(45)</sup>. Conversely, modulation of gut flora by antibiotics reduced metabolic endotoxaemia in diet-induced and the genetically obese *ob/ob* mice<sup>(50)</sup>. The binding of LPS to TLR4 on enterocytes triggers its phagocytosis and it is subsequently translocated across the intestinal barrier and into the lymph; from there LPS is packaged into chylomicrons along with dietary lipids and transported around the body<sup>(51)</sup>. Indeed, one study conducted in fasting normal-weight, healthy individuals has found that while intake of water, orange juice or a glucose drink has no effect on plasma endotoxin concentrations, consumption of cream increased plasma LPS levels<sup>(52)</sup>. It has also been shown in the genetically obese mouse models, *ob/ob* and *db/db*, that high-fat feeding and LPS

can increase intestinal wall permeability, elevating endotoxin levels in the portal circulation<sup>(50,53)</sup>. Recent research suggests that it is gut microbiota that mediate this effect, by modulating the intestinal endocannabinoid system<sup>(54)</sup>. In obese mice, blockade of the endocannabinoid receptor, CB<sub>1</sub> led to a reduction in gut permeability with a concomitant reduction in plasma LPS, which was also seen following prebiotic administration<sup>(54)</sup>.

Although it has recently been shown that tea catechins such as epigallocatechin-3-O-gallate (EGCG)<sup>(55)</sup>, anthocyanins and anthocyanidins<sup>(56)</sup> have a moderate affinity for endocannabinoid receptors at concentrations <50 μM, there is, as yet, no research into their ability to modulate gut permeability and thereby reduce plasma LPS levels. Furthermore, limited research has been conducted investigating their ability to limit the increases in LPS found following a high-energy meal. A small study using normal-weight, healthy subjects investigated plasma changes in endotoxin levels, inflammation and oxidative stress following consumption of a high-fat, high-carbohydrate meal with water, a glucose drink or orange juice<sup>(57)</sup>. Consumption of orange juice abrogated the increase in plasma endotoxin, inflammation and oxidative stress which was seen with glucose drink and water intake<sup>(57)</sup>. In a similar study, healthy, normal-weight individuals were given a nutritional supplement containing resveratrol (major polyphenol of red-wine grapes) and muscadine grape polyphenols along with a high-fat, high-carbohydrate meal. The supplement ameliorated the increase in plasma LPS-binding protein and endotoxin concentrations associated with intake of such a meal, along with reducing monocytic expression of TLR4<sup>(58)</sup>. The results of these studies indicate the merit of further research into this area and due to the extended post-prandial inflammatory response seen in obesity, should be extended to include this population.

TLR are transmembrane proteins that recognise microbial components such as bacterial LPS and flagellin and also possibly endogenous ligands released during inflammation<sup>(59)</sup>. TLR4 is thought to mediate cellular response to LPS, and TLR2 is regarded as the main receptor for Gram-positive bacterial and fungal cell wall components<sup>(60)</sup>; however, LPS activation of TLR4 on 3T3-L1 murine adipocytes has been found to induce the expression of TLR2, leading to an increase in IL-6 gene expression<sup>(60)</sup>. TLR expression has been observed in AT and adipocytes from normal-weight and obese human subjects and these are responsive to LPS, resulting in the activation of NF-κB signalling, which regulates immune and inflammatory responses, and subsequent cytokine release<sup>(61–63)</sup>. In mice fed a high-fat diet, knockdown of TLR ameliorated the increase in adiposity, reducing serum insulin concentrations and circulating markers of inflammation, although the mechanisms behind this are not fully understood<sup>(64)</sup>.

The bioactive xanthenes of the mangosteen fruit, α- and γ-mangostin, have recently been found to abrogate the activation of NF-κB signalling following incubation with LPS, with a concomitant reduction in pro-inflammatory gene expression in human adipocytes, *ex vivo*<sup>(65)</sup>. Furthermore, α-tocopherol treatment of 3T3-L1 cells stimulated with LPS abrogated production of the cytokine, IL-6<sup>(66)</sup>. Plant compounds have also been found to reduce

LPS-activation of immunocytes. Stimulation of the human acute monocytic leukemia cell line THP-1 with LPS increased the expression and secretion of TNF $\alpha$  and IL-6, which was significantly reduced by treatment with the common polyphenol, cyanidin 3-O- $\beta$ -glucoside<sup>(67)</sup>. EGCG is also known to be anti-inflammatory and abrogates the macrophage inflammatory response to LPS. Recent research suggests that this is mediated by its ability to inhibit TLR signalling by down-regulating TLR4 expression<sup>(68)</sup>.

### NEFA

Elevated plasma NEFA are a feature of obesity thought to result from increased AT basal lipolytic activity<sup>(69)</sup>, which is in turn due to increased adipocyte size<sup>(70)</sup> and cellular hypoxia<sup>(71)</sup>. Historically, increased plasma NEFA have been connected with obesity<sup>(72)</sup> and T2DM<sup>(73)</sup> although further investigations have failed to find any significant associations<sup>(74)</sup>. Regardless, there is much research to indicate the ability of NEFA to impair the insulin responsiveness of tissues including the muscle and liver, which is thought to result from the inhibition of GLUT translocation caused by the intracellular accumulation of fatty acid metabolites<sup>(75)</sup>. The polyphenolic compounds, EGCG<sup>(76)</sup> and naringenin<sup>(77)</sup>, have been shown to increase GLUT translocation in rat L6 skeletal muscle cells, thereby enhancing glucose uptake; this has also been found in 3T3-L1 adipocytes treated with the phenolic acid, gallic acid, derived from seabuckthorn<sup>(78)</sup>. Conversely, in isolated rat adipocytes, the polyphenols catechin-gallate, myricetin and quercetin, widely present in fruits and vegetables, have been shown to directly interact with GLUT4, reducing glucose transport<sup>(79)</sup>; however, this has not been replicated *in vivo*. In a fructose-fed rat model of insulin resistance, AT concentrations of GLUT4 were reduced by 58% compared to the control group; however, in those supplemented with green-tea powder, GLUT4 levels were not significantly different from the controls (19%) thereby increasing insulin sensitivity<sup>(80)</sup>. Further to this, the induction of insulin resistance in rats by 48 h intravenous infusion of a TAG emulsion, inhibited the translocation of GLUT4 in both adipose and skeletal muscle tissue. However, this was abrogated in both tissues with the co-injection of 10 mg/kg EGCG; additionally there was a concomitant improvement in insulin-induced glucose uptake<sup>(81)</sup>.

Recently it has been found that normal fasting NEFA levels are maintained in abdominally obese men through a reduction in lipolytic enzyme expression and therefore reduced fasting state NEFA release<sup>(82)</sup>. However, it was further found that post-prandial fatty acid uptake by AT was significantly reduced, which is attributed to a down-regulation of fat storage mechanisms and resulted in a substantial increase in plasma NEFA following a meal<sup>(82)</sup>. This is proposed to lead to an increase in ectopic fat deposition; however, this requires further research<sup>(82)</sup>. Phytochemicals such as luteolin, a flavonoid found in olive oil and carrots<sup>(83)</sup>, resveratrol<sup>(84)</sup> and theaflavins found in black tea<sup>(85)</sup> have been found to limit lipid accumulation in human liver HepG2 cells. This has also been found with curcumin treatment of human Hep3B cells<sup>(86)</sup> and EGCG

treatment of primary mouse hepatocytes<sup>(87)</sup>. The proposed mechanism of action is thought to be activation of AMP-activated protein kinase  $\alpha$  signalling pathway, which subsequently inhibits fatty acid and cholesterol synthesis and up-regulates fatty acid oxidation and glycolysis, thereby regulating cellular energy balance<sup>(88)</sup>.

There are a number of animal studies indicating the benefits of phytochemicals in reducing liver lipotoxicity. Supplementation of the genetically obese KK/A<sup>y</sup> mice with rhaponticin, extracted from rhubarb, reduced plasma NEFA and TAG levels in addition to preventing the development of liver steatosis<sup>(89)</sup>. Theaflavin treatment of rats fed a high-fat diet ameliorated the development of liver steatosis with a concomitant reduction in plasma NEFA and TAG, which was shown to be mediated through activation of AMP-activated protein kinase signalling<sup>(85)</sup>. In a rat model of liver steatosis, where treatment with carbon tetrachloride induces liver fat accumulation and hepatic cell death, prior feeding with an apricot extract significantly attenuated this<sup>(90)</sup>. Olive leaf extract has also been shown to attenuate the development of liver steatosis in a high-fat fed rat model of obesity, with a concomitant reduction in plasma TAG; however, circulating NEFA levels were not affected<sup>(91)</sup>. Resveratrol supplementation of rats with diet-induced liver steatosis significantly reduced fat deposition in the liver<sup>(92)</sup>. None of these studies investigated alterations in adipose or muscle tissue gene expression of lipid-processing mechanisms, which given the recent findings in obese human subjects<sup>(82)</sup> would be pertinent. Furthermore, there are, as yet, few studies investigating their effects in human subjects.

Characterisation of fasting plasma NEFA in healthy, normal-weight individuals has found that SFA comprise just over half (56%) of the total NEFA concentration, and of this fraction palmitate predominates<sup>(93)</sup>. Further research has found that consumption of a saturated NEFA (sNEFA)-rich diet by abdominally obese participants for 8 weeks resulted in an increase in AT inflammatory gene expression compared to those on a MUFA-rich diet<sup>(94)</sup>. In cell culture, sNEFA have been shown to induce release of pro-inflammatory cytokines from macrophages<sup>(95,96)</sup> and in adipocytes, increase reactive oxygen species generation and TNF $\alpha$ , IL-6 and monocyte chemoattractant protein-1 gene expression along with reducing adiponectin gene expression<sup>(97-99)</sup>. TNF $\alpha$  has been found to decrease adipocyte insulin sensitivity, which increases lipolysis and therefore plasma levels of NEFA<sup>(100,101)</sup>. Even short-term exposure of adipocytes to TNF $\alpha$  stimulates lipolysis through increased inducible nitric oxide synthase expression, which in turn up-regulates nitric oxide production and subsequently phosphorylates and activates hormone-sensitive lipase<sup>(102)</sup>. Further research has found that immunocyte response to LPS, mediated by TLR4 activation, is approximately three-fold higher in those pre-treated with sNEFA than those treated with either sNEFA or LPS alone<sup>(103)</sup>. A positive feedback loop is proposed whereby sNEFA released from adipocytes activate macrophages, stimulating further release of TNF $\alpha$  and IL-6 and these in turn cause inflammatory changes in adipocytes<sup>(104)</sup>.

Studies *in vitro* have shown that sNEFA activate TLR2 and TLR4 on adipocytes and macrophages triggering c-Jun

N-terminal kinase and NF- $\kappa$ B pro-inflammatory signalling cascades resulting in pro-inflammatory cytokine production<sup>(105–108)</sup>. However, recent research suggests that it may be LPS contamination of the fatty acid-free bovine serum albumin, a reagent commonly used to complex with sNEFA in *in vitro* studies, which stimulates TLR4 as uncomplexed sNEFA had no effect on TLR-dependent signalling<sup>(109)</sup>. Although this is a matter that requires further clarification, numerous cell and animal studies do show that sNEFA are able to elicit a pro-inflammatory response; furthermore, these low levels of LPS may better represent the *in vivo* milieu<sup>(110)</sup>. There are, as yet, limited studies investigating the inhibitory effects of phytochemicals on the pro-inflammatory response of macrophages or adipocytes to sNEFA. However, as mentioned previously, studies in both adipocytes and macrophages have shown that phytochemicals, such as  $\alpha$ - and  $\gamma$ -mangostin<sup>(65)</sup>,  $\alpha$ -tocopherol<sup>(66)</sup> and EGCG<sup>(68)</sup>, are able to inhibit signalling pathways downstream of LPS-mediated TLR activation, ameliorating pro-inflammatory gene expression.

### Adipocyte hypertrophy

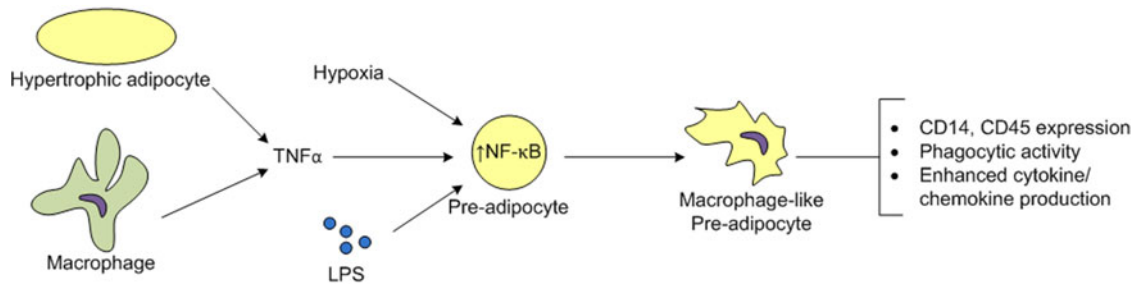
Hypertrophic adipocytes have been associated with hypertension<sup>(111)</sup> and an increased risk of CVD<sup>(112)</sup>, and further research in human subjects and mice has found that adipocyte size positively correlates with degree of AT inflammation<sup>(113–116)</sup>. Hypertrophic adipocytes isolated from human subjects have been found to have altered gene expression<sup>(117)</sup> leading to increased secretion of pro-inflammatory factors and altered adipokine secretion<sup>(118–120)</sup>. Anti-obesity research has investigated the ability of nutraceuticals to promote adipocyte apoptosis and inhibit adipocyte differentiation, thereby reducing AT accumulation. The two most well-studied transcription factors regulating adipogenesis are PPAR $\gamma$  and cytidine-cytidine-adenosine-adenosine-thymidine-enhancer-binding proteins (C/EBP)<sup>(121)</sup>. PPAR $\gamma$  is considered to be essential and adequate for the induction of adipocyte differentiation and has two isoforms, PPAR $\gamma$ 1 and PPAR $\gamma$ 2, whose distinct roles are yet to be elucidated. Members of the C/EBP family are either anti- or pro-adipogenic and include C/EBP $\alpha$ , C/EBP $\beta$ , C/EBP $\gamma$  and C/EBP $\delta$ . Their expression is necessary for the development of adipocyte insulin sensitivity<sup>(122)</sup> and while they are important for adipogenesis, the presence of PPAR $\gamma$  is still required.

The anti-adipogenic potential of various natural compounds has been investigated and many have been found to inhibit adipocyte differentiation. The addition of curcumin, the major polyphenol found in turmeric, to rodent models of obesity has been shown to reduce body weight gain and ameliorate the development of diabetes<sup>(123,124)</sup>. This is thought to be due to its ability to suppress adipocyte differentiation and indeed this has been found in studies using the murine 3T3-L1 cell line, with a concomitant down-regulation of PPAR $\gamma$ <sup>(125,126)</sup>; however, in another study utilising 3T3-L1 cells, these transcriptional markers were not affected<sup>(124)</sup>. A recent comprehensive investigation into the ligand activity of curcumin found it to have no binding activity at the PPAR $\gamma$  ligand-binding site and subsequently

no effect on adipocyte differentiation<sup>(127)</sup>. These disparities in curcumin activity are most likely related to the different extractions used as some studies have utilised purified curcumin, while others have prepared ethanolic extracts of turmeric which may contain other active compounds.

The green-tea polyphenol, EGCG, induces cancer cell apoptosis and has been shown to increase weight loss and reduce fat accumulation in both human<sup>(128)</sup> and rodent obesity studies<sup>(129,130)</sup>. In one study, administration of EGCG to the diets of high-fat fed rats abrogated the development of glucose intolerance with a concomitant increase in PPAR $\gamma$  gene expression; however, contrastingly, administration of green tea suppressed PPAR $\gamma$  gene expression<sup>(131)</sup>. In 3T3-L1 cells, investigations have attributed the anti-obesity effects of EGCG to its ability to induce adipocyte apoptosis and inhibit adipocyte differentiation and proliferation, through down-regulation of PPAR $\gamma$  and C/EBP $\alpha$ <sup>(132–134)</sup>. The concentrations used in these studies ranged from 0 to 200  $\mu$ M; however, one study found that EGCG administration at 0.5–10  $\mu$ M, while having no effect on cell activity, enhanced expression of genes related to adipocyte differentiation and insulin sensitivity and reduced fat accumulation<sup>(135)</sup>. In human subjects, studies investigating the bioavailability of green-tea polyphenols following consumption of green-tea solids in water have found the maximum plasma concentration of EGCG to be <1  $\mu$ g/ml<sup>(136,137)</sup>; therefore, studies carried out in cells using lower levels of EGCG may better mimic physiological levels.

However, is the inhibition of adipocyte differentiation truly beneficial? Recent research has indicated that in the moderately obese (BMI 26–36 kg/m<sup>2</sup>), an increase in the number of smaller adipocytes may also contribute to AT inflammation. In the study, abdominal AT biopsies were taken from healthy, moderately obese individuals and analysed for cell size distribution and inflammatory gene expression<sup>(138)</sup>. Adipocyte size was not found to be associated with inflammatory gene expression, instead an increase in the proportion of smaller adipocytes predicted the expression of inflammatory genes, which was independent from sex, insulin resistance and BMI, although this association was stronger in insulin-resistant than insulin-sensitive individuals<sup>(138)</sup>. A total of eight inflammatory genes were analysed and of these CD14 and CD45 are specific for monocyte lineage cells, suggesting the acquisition of a monocytic phenotype by the smaller adipocytes. Previous research has found that pre-adipocytes acquire functional properties similar to macrophages when cultured in contact with one another and their gene profile has been found to be closer to macrophages than adipocytes<sup>(139)</sup>. As outlined in Fig. 2, there are many factors in pathogenic AT which may affect the normal differentiation of pre-adipocytes; this then results in the development of a pro-inflammatory, macrophage-like phenotype as indicated by the elevated secretion of cytokines and chemokines<sup>(140,141)</sup>, most likely by activation of the key transcriptional regulator of inflammation, NF- $\kappa$ B<sup>(141)</sup>. Further research in subcutaneous AT biopsies obtained from obese individuals suggests that there is a failure of pre-adipocytes to differentiate<sup>(140,141)</sup>, possibly caused by elevated levels of TNF $\alpha$ <sup>(140)</sup>.



**Fig. 2.** (Colour online) Factors contributing to conversion of pre-adipocyte to one with macrophage-like activity. LPS, lipopolysaccharide.

LPS, high circulating levels of which are found in the obese or those following a high-fat meal, has also been found to affect adipocyte differentiation. Pre-adipocytes are known to have a macrophage-like phenotype and have a high gene expression and secretion of pro-inflammatory cytokines<sup>(142,143)</sup> in response to TNF $\alpha$  and LPS<sup>(139,144)</sup>. IL-6 expression levels were found to be higher in LPS-stimulated 3T3-L1 pre-adipocytes than in mature 3T3-L1 adipocytes<sup>(145)</sup>, and continuous treatment of these cells with LPS impaired their differentiation<sup>(146)</sup>. Furthermore, in primary cultures of human adipocytes, LPS stimulation up-regulated pro-inflammatory cytokine mRNA, predominantly in the pre-adipocyte fraction and, as differentiation into mature adipocytes occurred pro-inflammatory cytokine expression decreased<sup>(144)</sup>. Moreover, the therapeutic effects of thiazolidinediones used in the treatment of diabetes have been attributed, in part, to their ability to increase adipocyte differentiation<sup>(147)</sup>. Treatment of genetically obese Zucker rats with the thiazolidinedione, troglitazone normalised hyperglycaemia and hyperinsulinaemia in addition to reducing AT levels of TNF $\alpha$  and leptin<sup>(1)</sup>. This was concomitant with an increase in the number of small adipocytes and a reduced number of large adipocytes, without alterations to the total weight of white AT<sup>(148)</sup>.

Supplementation of diet-induced obese rats with bitter melon extract powder or the thiazolidinedione, pioglitazone, prevented the development of hyperinsulinaemia and glucose intolerance<sup>(149)</sup>. The number of large adipocytes (>180  $\mu$ m) was significantly lower in these two groups compared with those on the high-fat diet alone. In addition, the number of smaller adipocytes (60–100  $\mu$ m) in the bitter melon group was similar to those fed the low-fat diet, which was significantly higher than the high-fat and thiazolidinedione-treated groups. Bitter melon powder had the added effect of reducing AT mass and lipid content, suggesting that lipogenesis within AT was also attenuated<sup>(149)</sup>. *In vitro* studies have identified further compounds able to enhance adipocyte differentiation. Phloretin, a flavonoid found in apples and strawberries, was found to increase TAG accumulation with an attendant up-regulation of PPAR $\gamma$  and C/EBP $\alpha$ , concomitantly increases in adiponectin expression and secretion were also found<sup>(150)</sup>. Found in citrus fruits, the flavone nobiletin has been shown to enhance 3T3-L1 differentiation; however, this was not due to any activity at PPAR $\gamma$ . Instead, nobiletin activated C/EBP $\beta$ , which is up-stream of PPAR $\gamma$  and induces its

expression<sup>(151)</sup>. Further research clearly needs to be undertaken to fully determine the impact of individual cell populations within AT on adipose inflammation; however, it can be seen that failure of pre-adipocytes to differentiate can be detrimental with respect to either overloading of mature adipocytes rendering them hypertrophic or increasing the inflammatory pre-adipocyte population.

#### Adipose tissue hypoxia

Secondary to the development of hypertrophic adipocytes is AT hypoxia. Adipocytes can expand up to 150–200  $\mu$ m in diameter<sup>(118)</sup> and the maximum diffusion distance of oxygen is 200  $\mu$ m; therefore adipocyte hypertrophy can lead to AT hypoxia as their size may impair oxygen diffusion into the cell<sup>(4)</sup>. A recent study in obese human subjects has shown their abdominal AT to have a lower capillary density and oxygen partial pressure than lean subjects and this correlated negatively with percentage body fat and macrophage inflammatory protein-1 secretion, which is involved in inflammatory cell recruitment and release of pro-inflammatory cytokines<sup>(152)</sup>. Furthermore, the increase in AT blood flow associated with the post-prandial state is not seen in the obese<sup>(82)</sup>. AT hypoxia is also found in rodent models of obesity, in addition to increased expression of hypoxia-regulated genes and dysregulated adipokine secretion<sup>(71,153,154)</sup>. Hypoxia-inducible factor (HIF)-1 $\alpha$  and HIF-2 $\alpha$  are transcription factors induced by hypoxia that affect angiogenesis, glycolysis, cell proliferation, apoptosis and inflammation<sup>(155,156)</sup>. Gene expression of HIF-1 $\alpha$  has been positively correlated with body mass<sup>(157)</sup> and in adipocytes, HIF-1 $\alpha$  and HIF-2 $\alpha$  accumulation have been shown to promote the development of an insulin-resistant state by decreasing insulin receptor phosphorylation<sup>(158)</sup>. In adipocyte cell lines, hypoxia reduces NEFA uptake and increases lipolysis as well as inducing necrosis and apoptosis<sup>(71)</sup>, pro-inflammatory cytokine production is also increased and adipokine secretion dysregulated<sup>(159,160)</sup>. Furthermore, the differentiation of pre-adipocytes is inhibited<sup>(161)</sup>.

Production of angiogenic factors, such as vascular endothelial growth factor and platelet-derived growth factor, are increased in response to hypoxia<sup>(162,163)</sup> and circulating levels of these are found to be elevated in obese human subjects and mice<sup>(164,165)</sup>. A recent rodent study has shown that angiogenesis supports, and is essential for

adipogenesis and that they occur together in cell clusters; however, as fat mass increases hypertrophy dominates with a reduction in the occurrence of adipogenic/angiogenic cell clusters<sup>(166)</sup>. Nutraceuticals have been found to reduce the expression and activity of HIF in cancer cells<sup>(167)</sup> with the overall aim to reduce tumour angiogenesis and therefore growth; however, similar inhibition in AT may only serve to further exacerbate hypoxia. In ischaemia-reperfusion injury, the promotion of HIF and its downstream signalling targets is beneficial, limiting infarct size by stimulating angiogenesis<sup>(168)</sup>. The subunits of HIF, HIF-1 $\alpha$ , HIF-2 $\alpha$  and the recently discovered HIF-3 $\alpha$ , differentially regulate adipogenesis<sup>(169)</sup>; however, their exact roles are, as yet, ill defined. The role of HIF and its subunits in AT dysfunction is a recent area of research and needs to be further understood before investigations into its regulation by nutraceuticals can get underway.

### Adipocyte dysfunction

Researchers have attempted to establish what the fundamental triggering factor is that tips healthy AT towards one which is pro-inflammatory; one potential candidate is endoplasmic reticulum (ER) stress. Markers of ER stress occur in AT of both diet and genetically induced mouse models of obesity<sup>(170)</sup> and more recently in obese human subjects<sup>(171–173)</sup>, and have been found to be reduced by weight loss<sup>(173)</sup>. Recently, ER stress has been linked with reduced secretion of adiponectin, a key adipokine in the regulation of insulin sensitivity and inflammation in obesity. Incubation of 3T3-L1 adipocytes with a protease inhibitor induced ER stress which resulted in a reduction in adiponectin synthesis through the activation of c-Jun N-terminal kinase signalling pathway and subsequent induction of activating transcription factor 3<sup>(174)</sup>.

The ER is the principal site of protein synthesis within the cell, ensuring the transport and release of correctly folded proteins and within adipocytes, also facilitates lipid droplet formation<sup>(175–177)</sup>. An oxidative environment is present within the ER and is critical for disulfide bond formation and correct folding of proteins<sup>(177)</sup>. However, in reaction to disturbances such as nutrient deprivation, lipids and increased workload, unfolded proteins accumulate within the ER triggering the unfolded protein response (UPR), which results in reactive oxygen species accumulation and cellular oxidative stress<sup>(178)</sup>. This is mediated by three protein kinases, protein kinase RNA-like ER kinase, inositol-requiring 1 $\alpha$  and activating transcription factor 6<sup>(176,177)</sup>. As a transient measure, the UPR initially reduces protein synthesis and translocation into the ER, followed by a longer-term increase in the ability of the ER to handle unfolded proteins. If protein misfolding persists or is excessive then cell death is triggered, usually through apoptosis<sup>(176,177)</sup>.

The UPR is linked to inflammation through production and accumulation of reactive oxygen species, activation of the acute-phase response and activation of transcription factors regulating inflammatory signalling pathways such as NF- $\kappa$ B and c-Jun N-terminal kinase<sup>(175,179)</sup>. It is unknown as to what causes ER stress in obesity and there is little research into ER stress in the adipocyte; however,

some causative factors may include nutrient deprivation, resulting from decreased vascular density, with concomitant hypoxia, and increased protein synthesis due to adipocyte hypertrophy<sup>(180)</sup>. Elevated NEFA levels may also be a culprit, as these have been shown to activate the UPR in other cell types, including pancreatic  $\beta$ -cells and hepatocytes<sup>(181–183)</sup>. Conversely, ER stress may also result in elevated blood lipid levels, and research has found that protease inhibition in adipocytes, which induces ER stress, suppresses TAG synthesis and the transcription of lipogenic genes<sup>(184)</sup>.

Although antioxidants may promote ER stress by adversely affecting the oxidising environment of the ER, it has recently been shown that antioxidants could possibly have some benefit in reducing UPR-induced oxidative stress. One study investigated the expression of coagulation factor VIII, which is deficient in haemophilia A and is prone to misfolding in the ER<sup>(185)</sup>. It was found that factor VIII misfolding in the ER resulted in oxidative and ER stress *in vitro* in Chinese hamster ovary-(H9) cells with eventual apoptosis, which was attenuated by the addition of butylated hydroxyanisole, a phenolic, lipid-soluble antioxidant. An effect that was also mimicked *in vivo* in mice. Further to this, butylated hydroxyanisole treatment reduced the intracellular accumulation of misfolded factor VIII with a concomitant increase in functional factor VIII secretion, both *in vitro* and *in vivo*. Treatment of Chinese hamster ovary-H9 cells with ascorbic acid, which has weaker antioxidant properties than butylated hydroxyanisole, produced inconsistent results of less intensity<sup>(185)</sup>.

In another study, oxidative and ER stress were induced in human umbilical vein endothelial cells by incubation in hyperglycaemic conditions<sup>(186)</sup>. Interestingly though, while treatment with ascorbic acid and  $\alpha$ -tocopherol eliminated oxidative stress, no effect was found on ER stress, similar to the results found by Malhotra *et al.*<sup>(185)</sup>. This discrepancy may be due to the different cell lines used or, more likely, due to butylated hydroxyanisole having phenolic activity. Research in human colon cancer cell lines has shown that quercetin, a phenolic flavonoid, is able to reduce ER stress through the inhibition of the phosphoinositide 3-kinase pathway, which is not replicated by ascorbic acid or  $\alpha$ -tocopherol<sup>(187)</sup>. In a cell-free study, oxidative stress resulted in the loss of function of the ER protein-folding enzyme, protein disulfide isomerase causing an accumulation of misfolded proteins which was prevented by the addition of the polyphenolic compounds, curcumin and masoprocol<sup>(188)</sup>. While it has recently been shown that reduced adiponectin secretion from adipocytes is due, in part, to ER stress, it has yet to be investigated whether antioxidants could ameliorate ER stress in the adipocyte and, in so doing, possibly re-assert an anti-inflammatory secretory pattern. Given the evidence, it seems unlikely that a compound with purely antioxidant activity would have this effect, and therefore polyphenolic compounds may have more benefit.

### Conclusion

Despite the large amount of research into the development of pathogenic obesity, it is clear that this is a complex and

dynamic process and as such, further research is required in order to fully understand the mechanisms involved. There are thought to be four key areas that are disturbed during AT expansion leading to the initiation of inflammation within AT; metabolic endotoxaemia, increased plasma NEFA, hypertrophic adipocytes and increased AT hypoxia. Adipocyte ER stress is hypothesised to precede the development of the aforementioned four areas. A role for nutraceuticals in reversing the development of inflammation in obesity is a subject of great interest, and from the research reviewed here their potential in this area is clear. Although having been subject to intensive research in other areas, grape seed and green-tea polyphenols appear to also be particularly effective in pathogenic obesity, limiting metabolic endotoxaemia and the detrimental effects of elevated NEFA. Newly characterised compounds such as  $\alpha$ - and  $\gamma$ -mangostin and bitter melon extract were also found to have beneficial effects. However, as has been highlighted in this review, there is still much research that needs to be undertaken before the role of nutraceuticals in limiting the development of obesity-related comorbidities can be fully defined. In particular, further research in defined, obese populations using randomised controlled trials is essential.

Although initial research has focused on developing targeted interventions to limit the expansion of AT by reducing angiogenesis, inhibiting adipogenesis or promoting adipocyte apoptosis, this has a high risk of adverse side effects. It is, therefore, possibly more effective instead to direct research towards reversing the development of dysregulated AT activity in general, and in this respect, nutraceuticals may be the answer by providing a broader spectrum of treatment.

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